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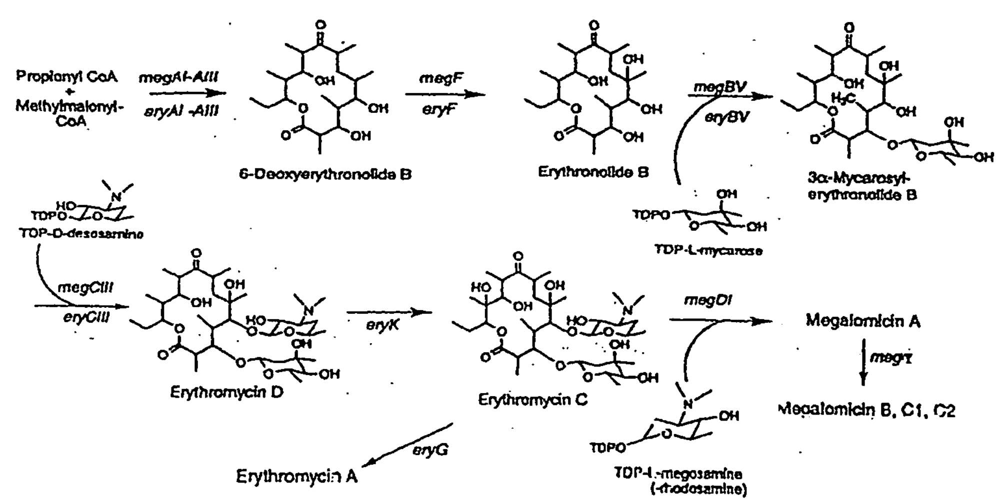
(54) Title: RECOMBINANT MEGALOMICIN BIOSYNTHETIC GENES AND USES THEREOF



(57) Abstract: Recombinant nucleic acid, *e.g* DNA compounds that encode all or a portion of the megalomicin polyketide synthase and modification enzymes are used to express recombinant polyketide synthase genes in host cells for the production of megalomicin, megalomicin derivatives, and other polyketides that are useful as antibiotics, motilides, and antiparasitics.

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Title

Recombinant Megalomicin Biosynthetic Genes And Uses Thereof

Cross-Reference to Priority Application

This application claims priority to provisional U.S. patent application Serial No. 60/158,305, filed 8 October 1999, and provisional U.S. patent application Serial No. 60/190,024, filed 17 March 2000 under 35 U.S.C. § 119(e). The content of the above referenced applications is incorporated herein by reference in its entirety.

Field of the Invention

The present invention provides recombinant methods and materials for producing polyketides by recombinant DNA technology. The invention relates to the fields of agriculture, animal husbandry, chemistry, medicinal chemistry, medicine, molecular biology, pharmacology, and veterinary technology.

Background of the Invention

Polyketides represent a large family of diverse compounds synthesized from 2-carbon units through a series of condensations and subsequent modifications. Polyketides occur in many types of organisms, including fungi and mycelial bacteria, in particular, the actinomycetes. There are a wide variety of polyketide structures, and the class of polyketides encompasses numerous compounds with diverse activities. Erythromycin, FK-506, FK-520, megalomicin, narbomycin, oleandomycin, picromycin, rapamycin, spinocyn, and tylosin are examples of such compounds. Given the difficulty in producing polyketide compounds by traditional chemical methodology, and the typically low production of polyketides in wild-type cells, there has been considerable interest in finding improved or alternate means to produce polyketide compounds. See PCT publication Nos. WO 93/13663; WO 95/08548; WO 96/40968; WO 97/02358; and WO 98/27203; United States Patent Nos. 4,874,748; 5,063,155; 5,098,837; 5,149,639; 5,672,491; and 5,712,146; Fu *et al.*, 1994, *Biochemistry 33*: 9321-9326; McDaniel *et al.*, 1993, *Science 262*: 1546-1550; and Rohr, 1995, *Angew.*

Chem. Int. Ed. Engl. *34*(8): 881-888, each of which is incorporated herein by reference.

Polyketides are synthesized in nature by polyketide synthase (PKS) enzymes. These enzymes, which are complexes of multiple large proteins, are similar to the synthases that catalyze condensation of 2-carbon units in the biosynthesis of fatty acids. PKS enzymes are encoded by PKS genes that usually consist of three or more open reading frames (ORFs). Two major types of PKS enzymes are known; these differ in their composition and mode of synthesis. These two major types of PKS enzymes are commonly referred to as Type I or "modular" and Type II "iterative" PKS enzymes.

Modular PKSs are responsible for producing a large number of 12-, 14-, and 16-membered macrolide antibiotics including erythromycin, megalomicin, methymycin, narbomycin, oleandomycin, picromycin, and tylosin. Each ORF of a modular PKS can comprise one, two, or more "modules" of ketosynthase activity, each module of which consists of at least two (if a loading module) and more typically three (for the simplest extender module) or more enzymatic activities or "domains." These large multifunctional enzymes (>300,000 kDa) catalyze the biosynthesis of polyketide macrolactones through multistep pathways involving decarboxylative condensations between acyl thioesters followed by cycles of varying ß-carbon processing activities (see O'Hagan, D. *The polyketide metabolites*; E. Horwood: New York, 1991, incorporated herein by reference).

During the past half decade, the study of modular PKS function and specificity has been greatly facilitated by the plasmid-based *Streptomyces coelicolor* expression system developed with the 6-deoxyerythronolide B (6-dEB) synthase (DEBS) genes (see Kao *et al.*, 1994, *Science, 265*: 509-512, McDaniel *et al.*, 1993, *Science 262*: 1546-1557, and U.S. Patent Nos. 5,672,491 and 5,712,146, each of which is incorporated herein by reference). The advantages to this plasmid-based genetic system for DEBS are that it overcomes the tedious and limited techniques for manipulating the natural DEBS host organism, *Saccharopolyspora erythraea*, allows more facile construction of recombinant PKSs, and reduces the complexity of PKS analysis by providing a "clean" host background. This system also expedited construction of the first combinatorial

modular polyketide library in *Streptomyces* (see PCT publication No. WO 98/49315, incorporated herein by reference).

The ability to control aspects of polyketide biosynthesis, such as monomer selection and degree of ß-carbon processing, by genetic manipulation of PKSs has stimulated great interest in the combinatorial engineering of novel antibiotics (see Hutchinson, 1998, *Curr. Opin. Microbiol. 1*: 319-329; Carreras and Santi, 1998, *Curr. Opin. Biotech. 9*: 403-411; and U.S. Patent Nos. 5,712,146 and 5,672,491, each of which is incorporated herein by reference). This interest has resulted in the cloning, analysis, and manipulation by recombinant DNA technology of genes that encode PKS enzymes. The resulting technology allows one to manipulate a known PKS gene cluster either to produce the polyketide synthesized by that PKS at higher levels than occur in nature or in hosts that otherwise do not produce the polyketide. The technology also allows one to produce molecules that are structurally related to, but distinct from, the polyketides produced from known PKS gene clusters.

Megalomicin is a macrolide antibiotic produced by *Micromonospora megalomicea*, a member of the Actinomycetales family of soil bacteria that produces many types of biologically active compounds. Megalomicin is a glycoside of erythromycin A, a widely used antibacterial drug with little or no antimalarial activity. Megalomicin has antibacterial properties similar to those of erythromycin, and in 1998, it was discovered also to have potent antiparasitic activity and low toxicity. The antiparasitic activity may be related to the effect megalomicin has on protein trafficking in eukaryotes, where it appears to inhibit vesicular transport between the medial and trans-Golgi, resulting in under-sialylation of proteins. Hence, megalomicin offers an exciting opportunity to develop a new class of antiparasitic drugs with a different mechanism of action than the drugs currently in use and, therefore, possibly active against drug-resistant forms of *Plasmodium falciparum*.

The number and diversity of megalomicin derivatives have been limited due to the inability to manipulate the PKS genes, which have not previously been available in recombinant form. Genetic systems that allow rapid engineering of the megalomicin biosynthetic genes would be valuable for creating novel compounds for pharmaceutical, agricultural, and veterinary applications. The production of

such compounds could be more readily accomplished if the heterologous expression of the megalomicin biosynthetic genes in *Streptomyces coelicolor* and *S. lividans* and other host cells were possible. The present invention meets these and other needs.

Summary of the Invention

The present invention provides recombinant methods and materials for expressing PKS enzymes and polyketide modification enzymes derived in whole and in part from the megalomicin biosynthetic genes in recombinant host cells. The invention also provides the polyketides produced by such PKS enzymes. The invention provides in recombinant form all of the genes for the proteins that constitute the complete PKS that ultimately results, in *Micromonospora megalomicea*, in the production of megalomicin. Thus, in one embodiment, the invention is directed to recombinant materials comprising nucleic acids with nucleotide sequences encoding at least one domain, module, or protein encoded by a megalomicin PKS gene. In one preferred embodiment of the invention, the DNA compounds of the invention comprise a coding sequence for at least one and preferably two or more of the domains of the loading module and extender modules 1 through 6, inclusive, of the megalomicin PKS.

In one embodiment, the invention provides a recombinant expression vector that comprises a heterologous promoter positioned to drive expression of one or more of the megalomicin biosynthetic genes. In a preferred embodiment, the promoter is derived from another PKS gene. In a related embodiment, the invention provides recombinant host cells comprising one or more expression vectors that produce(s) megalomicin or a megalomicin derivative or precursor. In a preferred embodiment, the host cell is *Streptomyces lividans* or *S. coelicolor*.

In another embodiment, the invention provides a recombinant expression vector that comprises a promoter positioned to drive expression of a hybrid PKS comprising all or part of the megalomicin PKS and at least a part of a second PKS. In a related embodiment, the invention provides recombinant host cells comprising the vector that produces the hybrid PKS and its corresponding polyketide. In a preferred embodiment, the host cell is *Streptomyces lividans* or *S. coelicolor*.

In a related embodiment, the invention provides recombinant materials for the production of libraries of polyketides wherein the polyketide members of the library are synthesized by hybrid PKS enzymes of the invention. The resulting polyketides can be further modified to convert them to other useful compounds, such as antibiotics, motilides, and antiparasitics, typically through hydroxylation and/or glycosylation. Modified macrolides provided by the invention that are useful intermediates in the preparation of antiparasitics are of particular benefit.

In another related embodiment, the invention provides a method to prepare a nucleic acid that encodes a modified PKS, which method comprises using the megalomicin PKS encoding sequence as a scaffold and modifying the portions of the nucleotide sequence that encode enzymatic activities, either by mutagenesis, inactivation, deletion, insertion, or replacement. The thus modified megalomicin PKS encoding nucleotide sequence can then be expressed in a suitable host cell and the cell employed to produce a polyketide different from that produced by the megalomicin PKS. In addition, portions of the megalomicin PKS coding sequence can be inserted into other PKS coding sequences to modify the products thereof.

In another related embodiment, the invention is directed to a multiplicity of cell colonies, constituting a library of colonies, wherein each colony of the library contains an expression vector for the production of a modular PKS derived in whole or in part from the megalomicin PKS. Thus, at least a portion of the modular PKS is identical to that found in the PKS that produces megalomicin and is identifiable as such. The derived portion can be prepared synthetically or directly from DNA derived from organisms that produce megalomicin. In addition, the invention provides methods to screen the resulting polyketide and antibiotic libraries.

The invention also provides novel polyketides, motilides, antibiotics, antiparasitics and other useful compounds derived therefrom. The compounds of the invention can also be used in the manufacture of another compound. In a preferred embodiment, the compounds of the invention are formulated in a mixture or solution for administration to an animal or human.

In a specific embodiment, the invention provides an isolated nucleic acid fragment comprising a nucleotide sequence encoding a domain of megalomicin polyketide synthase (PKS) or a megalomicin modification enzyme. The isolated

nucleic acid fragment can be a DNA or a RNA. Preferably, the isolated nucleic acid fragment is a recombinant DNA compound.

The isolated nucleic acid fragment can comprise a single, multiple or all the open reading frame(s) (ORF) of the megalomicin PKS or a megalomicin modification enzyme. Exemplary ORFs of megalomicin PKS include the ORFs of the *megAI*, *megAII* and *megAIII* genes. The isolated nucleic acid fragment can also encode a single, multiple, or all of the domains of the megalomicin PKS. Exemplary domains of the megalomicin PKS include a TE domain, a KS domain, an AT domain, an ACP domain, a KR domain, a DH domain and an ER domain. In a preferred embodiment, the nucleic acid fragment encodes a module of the megalomicin PKS. In another preferred embodiment, the nucleic acid fragment encodes the loading module, a thioesterase domain, and all six extender modules of the megalomicin PKS.

Megalomicin modification enzymes include those enzymes involved in the conversion of 6-dEB into a megalomicin such as the enzymes encoded by the *megF*, *meg BV*, *megCIII*, *megK*, *megDI* and *megG* (renamed *megY*) genes. Megalomicin modification enzymes also include those enzymes involved in the biosynthesis of mycarose, megosamine or desosamine, which are used as biosynthetic intermediates in the biosynthesis of various megalomicin species and other related polyketides. The enzymes that are involved in biosynthesis of mycarose, megosamine or desosamine are described in Figures 5 and 10.

In a preferred embodiment, the invention provides an isolated nucleic acid fragment which hybridizes to a nucleic acid having a nucleotide sequence set forth in the SEQ. ID NO:1, under low, medium or high stringency. More preferably, the nucleic acid fragment comprises, consists or consists essentially of a nucleic acid having a nucleotide sequence set forth in the SEQ. ID NO:1.

In another specific embodiment, the invention provides a substantially purified polypeptide, which is encoded by a nucleic acid fragment comprising a nucleotide sequence encoding a domain of megalomicin polyketide synthase (PKS) or a megalomicin modification enzyme. The polypeptide can comprise a single domain, multiple domains or a full-length megalomicin PKS or megalomicin modification enzyme. Functional fragments, analogs or derivatives of the megalomicin PKS or megalomicin modification enzyme polypeptides are

also provided. Preferably, such fragments, analogs or derivatives can be recognized by an antibody raised against a megalomicin PKS or megalomicin modification enzyme. Also preferably, such fragments, analogs or derivatives comprise an amino acid sequence that has at least 60% identity, more preferably at least 90% identity, to their wild type counterparts.

In still another specific embodiment, the invention provides an antibody, or a fragment or derivative thereof, which immuno-specifically binds to a domain of megalomicin polyketide synthase (PKS) or a megalomicin modification enzyme. The antibody can be a monoclonal or polyclonal antibody or an antibody fragment. Preferably, the antibody is a monoclonal antibody.

In yet another specific embodiment, the invention provides a recombinant DNA expression vector comprising the recombinant DNA compound encoding at least a domain of the megalomicin PKS or a megalomicin modification enzyme, wherein said domain is operably linked to a promoter. Preferably, the recombinant DNA expression vector further comprises an origin of replication or a segment of DNA that enables chromosomal integration.

In yet another specific embodiment, the invention provides a recombinant host cell comprising the above-described recombinant DNA expression vector encoding at least a domain of megalomicin PKS or the megalomicin modification enzyme. The recombinant host cells can be any suitable host cells including animal, mammalian, plant, fungal, yeast, and bacterial cells. Preferably, the recombinant host cells are *Streptomyces* cells, such as *Streptomyces lividans* and *S. coelicolor* cells, or *ccharopolyspora* cells, such as *Saccharopolyspora erythraea* cells. Also preferably, the recombinant host cells do not produce megalomicin in their untransformed, non-recombinant state.

When the recombinant host cell contains nucleic acid encoding more than one megalomicin PKS or megalomicin modification enzyme, or domains thereof, such nucleic acid material can be located at a single genetic locus, *e.g.*, on a single plasmid or at a single chromosomal locus, or at different genetic loci, *e.g.*, on separate plasmids and/or chromosomal loci. In one example, the invention provides a recombinant host cell, which comprises at least two separate autonomously replicating recombinant DNA expression vectors, and each of said vectors comprises a recombinant DNA compound encoding a megalomicin PKS

domain or a megalomicin modification enzyme operably linked to a promoter. In another example, the invention provides a recombinant host cell, which comprises at least one autonomously replicating recombinant DNA expression vector and at least one modified chromosome, each of said vector(s) and each of said modified chromosome comprises a recombinant DNA compound encoding a megalomicin PKS domain or a megalomicin modification enzyme operably linked to a promoter. Preferably, the autonomously replicating recombinant DNA expression vector and/or the modified chromosome further comprises distinct selectable markers.

In a preferred embodiment, the cell comprises three different vectors, one of which is integrated into the chromosome and two of which are autonomously replicating, and each of the vectors comprises a *meg* PKS gene. Optionally, one or more of the *meg* PKS genes contains one or more domain alterations, such as a deletion or substitution of a meg PKS domain with a domain from another PKS.

In yet another specific embodiment, the invention provides a hybrid PKS, which is produced from a recombinant gene that comprises at least a portion of a megalomicin PKS gene and at least a portion of a second PKS gene for a polyketide other than megalomicin. For example, and without limitation, the second PKS gene can be a narbonolide PKS gene, an oleandolide PKS gene, or a rapamycin PKS gene. In one embodiment, the hybrid PKS is composed of a loading module and six extender modules, wherein at least one domain of any one of extender modules 1 through 6, inclusive, is a domain of an extender module of megalomicin PKS. In another preferred embodiment, the hybrid PKS comprises a megalomicin PKS that has a non-functional KS domain in module 1.

In yet another specific embodiment, the invention provides a method of producing a polyketide, which method comprises growing the recombinant host cell comprising a recombinant DNA expression vector encoding at least a domain of the megalomicin PKS or a megalomicin modification enzyme under conditions whereby the megalomicin PKS domain or the megalomicin modification enzyme comprised by the recombinant expression vector is produced and the polyketide is synthesized by the cell, and recovering the synthesized polyketide. Preferably, the recombinant host cell comprises a recombinant expression vector that encodes at least a portion of a *megAI*, *megAII*, or *megAIII* gene.

These and other embodiments of the invention are described in more detail in the following description, the examples, and claims set forth below.

Brief Description of the Figures

Figure 1 shows restriction site and function maps of the insert DNA in cosmids pKOS079-138B, pKOS079-93D, pKOS079-93A, and pKOS079-124B of the invention. Various restriction sites (*Xho*I, *Bgl*II, *Nsi*I) are also shown. The location of the megalomicin biosynthetic genes is shown below the solid lines indicating the cosmid inserts. The genes are shown as arrows pointing in the direction of transcription. The approximate size (in kilobase (kb) pairs) of the gene cluster is indicated in 5000 bp (i.e., 5K, 10K, and the like.) increments on a solid bar beneath the arrows indicating the genes.

Figure 2 shows a more detailed map of the megalomicin biosynthetic gene cluster. The various open reading frames are shown as arrows pointing in the direction of transcription. A line indicates the size in base pairs (in 1000 bp increments) of the gene cluster. The various domains of the megalomicin PKS are also shown. Other genes of the megalomicin biosynthetic gene cluster not shown in this Figure are located in the insert DNA of cosmids pKOS0138B and pKOS0124B.

Figure 3 shows the structures of the megalomicins, azithromycin and erythromycin A.

Figure 4 shows the modules and domains of DEBS and the megalomicin PKS.

Figure 5 shows the compounds and reactions in the erythromycin biosynthetic pathway and also for megalomicin biosynthesis. Genes that produce the various enzymes that catalyze each of the steps in the biosynthetic pathway are indicated.

Figure 6 shows the biosynthetic pathway for the formation of desosamine, rhodosamine, and mycarose, as well as the genes that produce the various enzymes that catalyze each of the steps in the biosynthetic pathway.

Figure 7 depicts nucleotide and amino acid sequence of *Micromonospora megalomicea* megalomicin biosynthetic genes (GenBank Accession No. AF263245, incorporated herein by reference).

Figure 8 depicts the biosynthesis of the erythromycins and megalomicins and the enzymes that mediate the biosynthesis of each.

Figure 9 depicts the cloned megalomicin biosynthetic gene cluster and certain cosmids of the invention that comprise portions of the cluster.

Figure 10 depicts the biosynthesis of megosamine, mycarose, and desosamine.

Detailed Description of the Invention

The present invention provides useful compounds and methods for producing polyketides in recombinant host cells. As used herein, the term recombinant refers to a compound or composition produced by human intervention. The invention provides recombinant DNA compounds encoding all or a portion of the megalomicin biosynthetic genes. The invention provides recombinant expression vectors useful in producing the megalomicin PKS and hybrid PKSs composed of a portion of the megalomicin PKS in recombinant host cells. The invention also provides the polyketides produced by the recombinant PKS and polyketide modification enzymes.

To appreciate the many and diverse benefits and applications of the invention, the description of the invention below is organized as follows. In Section I, common definitions used throughout this application are provided. In Section II, structural and functional characteristics of megalomicin are described. In Section III, the recombinant megalomicin biosynthetic genes and other recombinant nucleic acids provided by the invention are described. In Section IV, polypeptides and proteins encoded by the megalomicin biosynthetic genes and antibodies that specifically bind to such polypeptides and proteins provided by the invention are described. In Section V, methods for heterologous expression of the megalomicin biosynthetic genes provided by the invention are described. In Section VI, the hybrid PKS genes provided by the invention are described. In Section VII, host cells containing multiple megalomicin biosynthetic genes and nucleic acid fragments on separate express vectors provided by the invention are described. In Section VIII, the polyketide compounds provided by the invention and pharmaceutical compositions of those compounds are described. The detailed description is followed by working examples illustrating the invention.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art to which this invention belongs. All patents, applications, published applications and other publications and sequences from GenBank and other data bases referred to herein are incorporated by reference in their entirety.

Section I. Definitions

As used herein, domain refers to a portion of a molecule, *e.g.*, proteins or nucleic acids, that is structurally and/or functionally distinct from another portion of the molecule.

As used herein, antibody includes antibody fragments, such as Fab fragments, which are composed of a light chain and the variable region of a heavy chain.

As used herein, biological activity refers to the *in vivo* activities of a compound or physiological responses that result upon *in vivo* administration of a compound, composition or other mixture. Biological activity, thus, encompasses therapeutic effects and pharmaceutical activity of such compounds, compositions and mixtures. Biological activities may be observed in in vitro systems designed to test or use such activities.

As used herein, a combination refers to any association between two or among more items.

As used herein, a composition refers to any mixture. It may be a solution, a suspension, liquid, powder, a paste, aqueous, non-aqueous or any combination thereof.

As used herein, derivative or analog of a molecule refers to a portion derived from or a modified version of the molecule.

As used herein, operably linked, operatively linked or operationally associated refers to the functional relationship of DNA with regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences. For example, operative linkage of DNA to a promoter refers to the physical and functional relationship between the DNA and the promoter such that the transcription of such DNA is initiated from the promoter by an RNA polymerase that specifically recognizes,

binds to and transcribes the DNA. To optimize expression and/or *in vitro* transcription, it may be helpful to remove, add or alter 5' untranslated portions of the clones to eliminate extra, potentially inappropriate alternative translation initiation (*i.e.*, start) codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites (see, *e.g.*, Kozak, *J. Biol. Chem.*, *266*:19867-19870 (1991)) can be inserted immediately 5' of the start codon and may enhance expression. The desirability of (or need for) such modification may be empirically determined.

As used herein, pharmaceutically acceptable salts, esters or other derivatives of the conjugates include any salts, esters or derivatives that may be readily prepared by those of skill in this art using known methods for such derivatization and that produce compounds that may be administered to animals or humans without substantial toxic effects and that either are pharmaceutically active or are prodrugs.

As used herein, a promoter region or promoter element refers to a segment of DNA or RNA that controls transcription of the DNA or RNA to which it is operatively linked. The promoter region includes specific sequences that are sufficient for RNA polymerase recognition, binding and transcription initiation. This portion of the promoter region is referred to as the promoter. In addition, the promoter region includes sequences that modulate this recognition, binding and transcription initiation activity of RNA polymerase. These sequences may be *cis* acting or may be responsive to *trans* acting factors. Promoters, depending upon the nature of the regulation, may be constitutive or regulated.

As used herein: stringency of hybridization in determining percentage mismatch is as follows: (1) high stringency: 0.1 x SSPE, 0.1% SDS, 65°C; (2) medium stringency: 0.2 x SSPE, 0.1% SDS, 50°C; and (3) low stringency: 1.0 x SSPE, 0.1% SDS, 50°C. Equivalent stringencies may be achieved using alternative buffers, salts and temperatures.

The term substantially identical or homologous or similar varies with the context as understood by those skilled in the relevant art and generally means at least 70%, preferably means at least 80%, more preferably at least 90%, and most preferably at least 95% identity.

As used herein, substantially identical to a product means sufficiently similar so that the property of interest is sufficiently unchanged so that the substantially identical product can be used in place of the product.

As used herein, isolated means that a substance is either present in a preparation at a concentration higher than that substance is found in nature or in its naturally occurring state or that the substance is present in a preparation that contains other materials with which the substance is not associated with in nature. As an example of the latter, an isolated meg PKS protein includes a meg PKS protein expressed in a *Streptomyces coelicolor* or *S. lividans* host cell.

As used herein, substantially pure means sufficiently homogeneous to appear free of readily detectable impurities as determined by standard methods of analysis, such as thin layer chromatography (TLC), gel electrophoresis and high performance liquid chromatography (HPLC), used by those of skill in the art to assess such purity, or sufficiently pure such that further purification would not detectably alter the physical and chemical properties, such as enzymatic and biological activities, of the substance. Methods for purification of the compounds to produce substantially chemically pure compounds are known to those of skill in the art. A substantially chemically pure compound may, however, be a mixture of stereoisomers or isomers. In such instances, further purification might increase the specific activity of the compound.

As used herein, vector or plasmid refers to discrete elements that are used to introduce heterologous DNA into cells for either expression or replication thereof. Selection and use of such vehicles are well known within the skill of the artisan. An expression vector includes vectors capable of expressing DNAs that are operatively linked with regulatory sequences, such as promoter regions, that are capable of effecting expression of such DNA fragments. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome.

Section II. Megalomicins

The megalomicins were discovered in 1969 at Schering Corp. as antibacterial agents produced by *Micromonospora megalomicea* (see Weinstein *et al.*, 1969, *J. Antibiotics 22*: 253-258, and U.S. Patent No. 3,632,750, both of which are incorporated herein by reference). Although the initial structural assignment was in error, a thorough reassessment of NMR data coupled with an X-ray crystal structure of a megalomicin A derivative (see Nakagawa and Omura, "Structure and Stereochemistry of Macrolides" in *Macrolide Antibiotics* (S. Omura, ed.), Academic Press, NY, 1984, incorporated herein by reference) established the structures shown in Figure 3. The megalomicins are 6-*O*-glycosides of erythromycin C with acetyl or propionyl groups esterified at the 3''' or 4''' hydroxyls of the mycarose sugar at the C-3-position. The C-6 sugar has been named "megosamine," although it had been identified 5 to 10 years earlier as L-rhodosamine or *N*-dimethyldaunosamine, deoxyamino sugars commonly present in the anthracycline antitumor drugs. The antibacterial potency, spectrum of activity, and toxicity (LD_{50} acute, 7-7.5 g/kg s.c. or oral; subacute, >500 mg/kg) of the megalomicins is similar to that of erythromycin A.

The megalomicins have two modes of biological activity. As antibacterials, they act like the erythromycins, which inhibit protein synthesis at the translocation step by selective binding to the bacterial 50*S* ribosomal RNA. They also affect

protein trafficking in eukaryotic cells (see Bonay *et al.*, 1996, *J. Biol. Chem.* *271*:3719-3726, incorporated herein by reference). Although the mechanism of action is not entirely clear, it appears to involve inhibition of vesicular transport between the medial and trans Golgi, resulting in under-sialylation of proteins. The megalomicins also strongly inhibit the ATP-dependent acidification of lysosomes *in vivo* (see Bonay *et al.*, 1997, *J. Cell. Sci. 110*:1839-1849, incorporated herein by reference) and cause an anomalous glycosylation of viral proteins, which may be responsible for their antiviral activity against herpes (Tox_{50}, 70-100 μM; see Alarcon *et al.*, 1984, *Antivir. Res.* 4:231-243, and Alarcon *et al.*, 1988, *FEBS Lett.* *231*:207-211, both of which are incorporated herein by reference).

Strikingly, the megalomicins are potent antiparasitic agents, showing an IC_{50} of 1 μg/ml in blocking intracellular replication of *Plasmodium falciparum* infected erythrocytes (see Bonay *et al.*, 1998, *Antimicrob. Agents Chemother.* *42*:2668-2673, incorporated herein by reference). The megalomicins are effective against *Trypanosoma cruzi* and *T. brucei* (IC_{50}, 0.2-2 μg/ml) plus *Leishmania donovani* and *L. major* promastigotes (IC_{50}, 3 and 8 μg/ml, respectively). Megalomicin is also active against the intracellular replicative, amastigote form of *T. cruzi*, completely preventing its replication in infected murine LLC/MK2 macrophages at a dose of 5 μg/ml. Importantly, the effective drug concentration is 500-fold less than the acute LD_{50} in mammals, and there is no toxicity to BALB/c mice at doses (50 mg/kg) that are completely curative for *T. brucei* infections. Because the erythromycins do not have such activity, although azithromycin (Figure 3) has been reported to be an effective acute and prophylactic treatment for malaria caused by *P. vivax* and *P. falciparum* (see Taylor *et al.*, 1999, *Clin. Infect. Dis.* 28:74-81, incorporated herein by reference), the antiparasitic action of the megalomicins is unique and probably related to the presence of the deoxyamino sugar megosamine at C-6 (Figure 3). Consequently, the megalomicins could be developed into potent antimalarial drugs with a high therapeutic index and be active against *P. falciparum* and other species that are resistant to currently used classes of antimalarials. They also could lead to potent antiparasitic agents against leishmaniasis, trypanosomiasis, and Chagas' disease. In view of the widespread use of the erythromycins and their good oral availability plus the low mammalian toxicity of macrolides in general, the megalomicins could be used prophylactically

to combat malaria, and as fermentation products, the megalomicins should be relatively inexpensive to produce.

The megalomicins belong to the polyketide class of natural products whose members have diverse structural and pharmacological properties (see Monaghan and Tkacz, 1990, *Annu. Rev. Microbiol. 44*: 271, incorporated herein by reference). The megalomicins are assembled by polyketide synthases through successive condensations of activated coenzyme-A thioester monomers derived from small organic acids such as acetate, propionate, and butyrate. Active sites required for condensation include an acyltransferase (AT), acyl carrier protein (ACP), and beta-ketoacylsynthase (KS). Each condensation cycle results in a ß-keto group that undergoes all, some, or none of a series of processing activities. Active sites that perform these reactions include a ketoreductase (KR), dehydratase (DH), and enoylreductase (ER). Thus, the absence of any beta-keto processing domain results in the presence of a ketone, a KR alone gives rise to a hydroxyl, a KR and DH result in an alkene, while a KR, DH, and ER combination leads to complete reduction to an alkane. After assembly of the polyketide chain, the molecule typically undergoes cyclization(s) and post-PKS modification (e.g. glycosylation, oxidation, acylation) to achieve the final active compound.

Macrolides such as erythromycin and megalomicin are synthesized by modular PKSs (see Cane *et al.*, 1998, *Science 282*: 63, incorporated herein by reference). For illustrative purposes, the PKS that produces the erythromycin polyketide (6-deoxyerythronolide B synthase or DEBS; see U.S. Patent No. 5,824,513, incorporated herein by reference) is shown in Figure 4. DEBS is the most characterized and extensively used modular PKS system. DEBS is particularly relevant to the present invention in that it synthesizes the same polyketide, 6-deoxyerythronolide B (6-dEB), synthesized by the megalomicin PKS. In modular PKS enzymes such as DEBS and the megalomicin PKS, the enzymatic steps for each round of condensation and reduction are encoded within a single "module" of the polypeptide (i.e., one distinct module for every condensation cycle). DEBS consists of a loading module and 6 extender modules and a chain terminating thioesterase (TE) domain within three extremely large polypeptides encoded by three open reading frames (ORFs, designated *eryAI*, *eryAII*, and *eryAIII*).

Each of the three polypeptide subunits of DEBS (DEBSI, DEBSII, and DEBSIII) contains 2 extender modules, DEBSI additionally contains the loading module. Collectively, these proteins catalyze the condensation and appropriate reduction of 1 propionyl CoA starter unit and 6 methylmalonyl CoA extender units. Modules 1, 2, 5, and 6 contain KR domains; module 4 contains a complete set, KR/DH/ER, of reductive and dehydratase domains; and module 3 contains no functional reductive domain. Following the condensation and appropriate dehydration and reduction reactions, the enzyme bound intermediate is lactonized by the TE at the end of extender module 6 to form 6-dEB.

More particularly, the loading module of DEBS consists of two domains, an acyl-transferase (AT) domain and an acyl carrier protein (ACP) domain. In other PKS enzymes, the loading module is not composed of an AT and an ACP but instead utilizes an inactivated KS, an AT, and an ACP. This inactivated KS is in most instances called KS^Q, where the superscript letter is the abbreviation for the amino acid, glutamine, that is present instead of the active site cysteine required for activity. The AT domain of the loading module recognizes a particular acyl-CoA (propionyl for DEBS, which can also accept acetyl) and transfers it as a thiol ester to the ACP of the loading module. Concurrently, the AT on each of the extender modules recognizes a particular extender-CoA (methylmalonyl for DEBS) and transfers it to the ACP of that module to form a thioester. Once the PKS is primed with acyl- and malonyl-ACPs, the acyl group of the loading module migrates to form a thiol ester (trans-esterification) at the KS of the first extender module; at this stage, extender module 1 possesses an acyl-KS and a methylmalonyl ACP. The acyl group derived from the loading module is then covalently attached to the alpha-carbon of the malonyl group to form a carbon-carbon bond, driven by concomitant decarboxylation, and generating a new acyl-ACP that has a backbone two carbons longer than the loading unit (elongation or extension). The growing polyketide chain is transferred from the ACP to the KS of the next module, and the process continues.

The polyketide chain, growing by two carbons each module, is sequentially passed as a covalently bound thiol ester from module to module, in an assembly line-like process. The carbon chain produced by this process alone would possess a ketone at every other carbon atom, producing a polyketone, from which the

name polyketide arises. Commonly, however, the beta keto group of each two-carbon unit is modified just after it has been added to the growing polyketide chain but before it is transferred to the next module by either a KR, a KR plus a DH, or a KR, a DH, and an ER. As noted above, modules may contain additional enzymatic activities as well.

Once a polyketide chain traverses the final extender module of a PKS, it encounters the releasing domain or thioesterase found at the carboxyl end of most PKSs. Here, the polyketide is cleaved from the enzyme and cyclyzed. The resulting polyketide can be modified further by tailoring or modification enzymes; these enzymes add carbohydrate groups or methyl groups, or make other modifications, i.e., oxidation or reduction, on the polyketide core molecule. For example, the final steps in conversion of 6-dEB to erythromycin A include the actions of a number of modification enzymes, such as: C-6 hydroxylation, attachment of mycarose and desosamine sugars, C-12 hydroxylation (which produces erythromycin C), and conversion of mycarose to cladinose via *O*-methylation, as shown in Figure 5.

With this overview of PKS and post-PKS modification enzymes, one can better appreciate the recombinant megalomicin biosynthetic genes provided by the invention and their function, as described in the following Section.

Section III: The Megalomicin Biosynthetic Genes and Nucleic Acid Fragments

The megalomicin PKS was isolated and cloned by the following procedure. Genomic DNA was isolated from a megalomicin producing strain of *Micromonospora megalomicea* subsp. nigra (ATCC 27598), partially digested with a restriction enzyme, and cloned into a commercially available cosmid vector to produce a genomic library. This library was then probed with probe generated from the erythromycin biosynthetic genes as well as from cosmids identified as containing sequences homologous to erythromycin biosynthetic genes. This probing identified a set of cosmids, which were analyzed by DNA sequence analysis and restriction enzyme digestion, which revealed that the desired DNA had been isolated and that the entire PKS gene cluster was contained in overlapping segments on four of the cosmids identified. Figure 1 shows the cosmids, and the portions of the megalomicin biosynthetic gene cluster in the

insert DNA of the cosmids. Figure 1 shows that the complete megalomicin biosynthetic gene cluster is contained within the insert DNA of cosmids pKOS079-138B, pKOS079-124B, pKOS079-93D, and pKOS079-93A. Each of these cosmids has been deposited with the American Type Culture Collection in accordance with the terms of the Budapest Treaty (cosmid pKOS079-138B is available under accession no. ATCC _____; cosmid pKOS079-124B is available under accession no. ATCC _____; cosmid pKOS079-93D is available under accession no. ATCC _____; and cosmid pKOS079-93A is available under accession no. ATCC _____). Various additional reagents of the invention can be isolated from these cosmids. DNA sequence analysis was also performed on the various subclones of the invention, as described herein. Further analysis of these cosmids and subclones prepared from the cosmids facilitated the identification of the location of various megalomicin biosynthetic genes, including the ORFs encoding the PKS, modules encoded by those ORFs, and coding sequences for megalomicin modification enzymes. The location of these genes and modules is shown on Figure 2.

Those of skill in the art will recognize that, due to the degenerate nature of the genetic code, a variety of DNA compounds differing in their nucleotide sequences can be used to encode a given amino acid sequence of the invention. The native DNA sequence encoding the megalomicin PKS and other biosynthetic enzymes and other biosynthetic enzymes of *Micromonospora megalomicea* is shown herein merely to illustrate a preferred embodiment of the invention, and the invention includes DNA compounds of any sequence that encode the amino acid sequences of the polypeptides and proteins of the invention. In similar fashion, a polypeptide can typically tolerate one or more amino acid substitutions, deletions, and insertions in its amino acid sequence without loss or significant loss of a desired activity. The present invention includes such polypeptides with alternate amino acid sequences, and the amino acid sequences encoded by the DNA sequences shown herein merely illustrate preferred embodiments of the invention.

The recombinant nucleic acids, proteins, and peptides of the invention are many and diverse. To facilitate an understanding of the invention and the diverse compounds and methods provided thereby, the following description of the various regions of the megalomicin PKS and the megalomicin modification

enzymes and corresponding coding sequences is provided. To facilitate description of the invention, reference to a PKS, protein, module, or domain herein can also refer to DNA compounds comprising coding sequences therefor and *vice versa*. Also, unless otherwise indicated, reference to a heterologous PKS refers to a PKS or DNA compounds comprising coding sequences therefor from an organism other than *Micromonospora megalomicea*. In addition, reference to a PKS or its coding sequence includes reference to any portion thereof.

Thus, the invention provides DNA molecules in isolated (i.e., not pure, but existing in a preparation in an abundance and/or concentration not found in nature) and purified (i.e., substantially free of contaminating materials or substantially free of materials with which the corresponding DNA would be found in nature) form. The DNA molecules of the invention comprise one or more sequences that encode one or more domains (or fragments of such domains) of one or more modules in one or more of the ORFs of the megalomicin PKS and sequences that encode megalomicin modification enzymes from the megalomicin biosynthetic gene cluster. Examples of PKS domains include the KS, AT, DH, KR, ER, ACP, and TE domains of at least one of the 6 extender modules and loading module of the three proteins encoded by the three ORFs of the megalomicin PKS gene cluster. Examples of megalomicin modification enzymes include those that synthesize the mycarose, desosamine, and megosamine moieties, those that transfer those sugar moieties to the polyketide 6-dEB, those that hydroxylate the polyketide at C-6 and C-12, and those that acylate the sugar moieties.

In an especially preferred embodiment, the DNA molecule is a recombinant DNA expression vector or plasmid, as described in more detail in the following Section. Generally, such vectors can either replicate in the cytoplasm of the host cell or integrate into the chromosomal DNA of the host cell. In either case, the vector can be a stable vector (i.e., the vector remains present over many cell divisions, even if only with selective pressure) or a transient vector (i.e., the vector is gradually lost by host cells with increasing numbers of cell divisions).

The megalomicin PKS gene cluster comprises three ORFs (*megAI*, *megAII*, and *megAIII*). Each ORF encodes two extender modules of the PKS; the first ORF also encodes the loading module. Each extender module is composed of at least a KS, an AT, and an ACP domain. The locations of the various encoding regions of

these ORFs are shown in Figure 2 and described with reference to the sequence information below. The megalomicin PKS produces the polyketide known as 6-dEB, shown in Figure 4. In megalomicin-producing organisms, 6-dEB is converted to erythromycin C by a set of modification enzymes. Thus, 6-dEB is converted to erythronolide B by the *megF* gene product (a homolog of the *eryF* gene product), then to 3-alpha-mycarosyl-erythronolide B by the *megBV* gene product (a homolog of the *eryBV* gene product), then to erythromycin D by the *megCIII* gene product (a homolog of the *eryCIII* gene product, then to erythromycin C by the *megK* gene product (a homolog of the *eryK* gene product).

In addition to these modification enzymes, such megalomicin-producing organisms also contain the modification enzymes necessary for the biosynthesis of the desosamine and mycarose moieties that are similarly utilized in erythromycin biosynthesis, as shown in Figure 5. Megalomicin A contains the complete erythromycin C structure, and its biosynthesis additionally involves the formation of L-megosamine (L-rhodosamine) and its attachment to the C-6 hydroxyl (Figures 3 and 5, inset), followed by acylation of the C-3''' and(or) C-4''' hydroxyls as the terminal steps. L-megosamine is the same as *N*-dimethyl-L-daunosamine; the daunosamine genes have been characterized from *Streptomyces peucetius* (see Colombo and Hutchinson, *J. Indust. Microbiol. Biotechnol.*, in press; Otten *et al.*, 1996, *J Bacteriol* 178:7316-7321, and references cited therein). Some of the rhodosamine genes also have been cloned and partially characterized from another anthracycline producing *Streptomyces* sp. (see Torkkell *et al.*, 1997, *Mol. Gen. Genet. 256*(2):203-209). Because the timing of the glycosylation with TDP-megosamine in relation to the addition of mycarose and desosamine to erythronolide B, plus the C-12 hydroxylation, is unknown, the pathway could involve a different order of glycosylation and C-12 hydroxylation steps than the one shown in Figure 5. Regardless, the megalomicin biosynthetic gene cluster contains the genes to make L-rhodosamine and attach it to the correct macrolide substrate.

The biosynthetic pathways to make the glycosides desosamine, mycarose, and megosamine are shown in Figure 6. The present invention provides the genes for each biosynthetic pathway shown in this Figure, and these recombinant genetic

pathways can be used alone or in any combination to confer the pathway to a heterologous host.

The megalomicin PKS locus is similar to the *eryA* locus in size and organization. Most of the deoxysugar biosynthesis genes are homologs of the *eryB* mycarose and *eryC* desosamine biosynthesis and glycosyl attachment genes from *Saccharopolyspora erythraea* (see Summers *et al.*, 1997, *Microbiol. 143*:3251-3262; Haydock *et al.*, 1991, *Mol. Gen. Genet.* 230:120-128; Gaisser *et al.*, 1997, *Mol Gen Genet*, 256:239-251; Gaisser *et al.*, 1998, *Mol Gen Genet.* 257:78-88, incorporated herein by reference) or the *picC* homologs from the picromycin and narbomycin producer (see PCT patent publication No. 99/61599 and Xue et al., 1998, *Proc. Nat. Acad. Sci. USA 95*, 12111-12116, incorporated herein by reference). The TDP-megosamine biosynthesis genes are homologs of the *dnm* genes (see Figure 5) and the pikromycin N-dimethyltransferase gene or its homologs reported in a cluster of L-rhodosamine biosynthesis genes. The putative TDP-megosamine glycosyltransferase gene product (*geneX* in Figure 5) closely resembles the deduced products of the *eryBV*, *eryCIII*, *dnmS*, and pikromycin *desVII* genes, even though it recognizes different substrates than the products of each of these genes.

The following Table 1 shows the location of the genes in the *Micromonospora megalomicea* megalomicin biosynthetic pathway in the DNA sequence set forth in SEQ ID NO:1 (see also Figure 7; note some gene designations maybe different in Figure 7).

Table 1. Megalomicin Biosynthetic Gene Cluster
Micromonospora megalomicea subsp. nigra (ATCC27598)

Location	Description
1..2451	sequence from cosmid pKOS079-138B
complement(1..144)	*megBVI (or megT)*, TDP-4-keto-6-deoxyglucose-2,3-dehydratase
928..2061	*megDVI*, TDP-4-keto-6-deoxyglucose 3,4-isomerase
2072..3382	*megDI*, TDP-megosaminyl transferase (*eryCIII* homolog)
2452..40397	sequence of cosmid pKOS079-93D
3462..4634	*megG(or megY)*, mycarosyl acyltransferase
4651..5775	*megDII*, deoxysugar transaminase (*eryCI*, *DnrJ* homolog)

5822..6595	*megDIII*, TDP-daunosaminyl-N,N-dimethyltransferase (*eryCVI* homolog)
6592..7197	*megDIV*, TDP-4-keto-6-deoxyglucose 3,5-epimerase (*eryBVII*, *dnmU* homolog)
7220..8206	*megDV*, TDP-hexose 4-ketoreductase (*eryBIV*, *dnmV* homolog)
complement(8228..9220)	*megBII*-1 or *megDVII*, TDP-4-keto-L-6-deoxy-hexose 2,3-reductase
complement(9226..10479)	*megBV*, TDP-mycarosyl transferase
complement(10483..11424)	*megBIV*, TDP-hexose 4-ketoreductase
12181..22821	*megAI*
12181..13791	Loading Module (L)
12505..13470	AT-L
13576..13791	ACP-L
13849..18207	Extender Module 1 (1)
13849..15126	KS1
15427..16476	AT1
17155..17694	KR1
17947..18207	ACP1
18268..22575	Extender Module 2 (2)
18268..19548	KS2
19876..20910	AT2
21517..22053	KR2
22318..22575	ACP2
22867..33555	*megAII*
22957..27258	Extender Module 3 (3)
22957..24237	KS3
24544..25581	AT3
26230..26733	KR3 (inactive)
26998..27258	ACP3
27313..33312	Extender Module 4 (4)
27393..28590	KS4
28897..29931	AT4
29953..30477	DH4
31396..32244	ER4
32257..32799	KR4
33052..33312	ACP4
33666..43271	*megAIII*
33780..38120	Extender Module 5 (5)
33780..35027	KS5
35385..36419	AT5
37068..37604	KR5
37860..38120	ACP5
38187..42425	Extender Module 6 (6)
38187..39470	KS6
39795..40811	AT6
40398..46641	sequences from cosmid pKOS079-93A

41406..41936	KR6
42168..42425	ACP6
42585..43271	TE
43268..44344	*megCII*, TDP-4-keto-6-deoxyglucose 3,4-isomerase
44355..45623	*megCIII*, TDP-desosaminyl transferase
45620..46591	*megBII*, TDP-4-keto-6-deoxy-L-glucose 2,3 dehydratase
complement(46660..47403)	*megH*, TEII
complement(47411..47980)	*megF*, C-6 hydroxylase

In a specific embodiment, the invention provides an isolated nucleic acid fragment comprising a nucleotide sequence encoding a domain of the megalomicin polyketide synthase or a megalomicin modification enzyme. The isolated nucleic acid fragment can be a DNA or a RNA. Preferably, the isolated nucleic acid fragment is a recombinant DNA compound. A nucleotide sequence that is complementary to the nucleotide sequence encoding a domain of megalomicin PKS or a megalomicin modification enzyme is also provided.

The isolated nucleic acid fragment can comprise a single, multiple or all the open reading frame(s) (ORF) of the megalomicin PKS or the megalomicin modification enzyme. Exemplary ORFs of megalomicin PKS include the ORFs of the *megAI*, *megAII* and *megAIII* genes. The isolated nucleic acids of the invention also include nucleic acids that encode one or more domains and one or more modules of the megalomicin PKS. Exemplary domains of the megalomicin PKS include a TE domain, a KS domain, an AT domain, an ACP domain, a KR domain, a DH domain and an ER domain. In a preferred embodiment, the nucleic acid comprises the coding sequence for a loading module, a thioesterase domain, and all six extender modules of the megalomicin PKS.

Megalomicin modification enzymes include those enzymes involved in the conversion of 6-DEB into a megalomicin such as the enzymes encoded by *megF*, *meg BV*, *megCIII*, *megK*, *megDI* and *megG* (or *megY*). Megalomicin modification enzymes also include those enzymes involved in the biosynthesis of mycarose, megosamine or desosamine, which are used as biosynthetic intermediates in the biosynthesis of various megalomicin species and other related polyketides. The enzymes that are involved in biosynthesis of mycarose, megosamine or desosamine are described in Figures 5 and 10. The megalomicin PKS and megalomicin modification enzymes are collectively referred to as megalomicin

biosynthetic enzymes; the genes encoding such enzymes are collectively referred to as megalomicin biosynthetic genes; and nucleic acids that comprise a portion of or entire megalomicin biosynthetic genes are collectively referred to as megalomicin biosynthetic nucleic acid(s).

In specific embodiments, the megalomicin biosynthetic nucleic acids comprise the sequence of SEQ ID NO:1, or the coding regions thereof, or nucleotide sequences encoding, in whole or in part, a megalomicin biosynthetic enzyme protein. The isolated nucleic acids typically consists of at least 25 (continuous) nucleotides, 50 nucleotides, 100 nucleotides, 150 nucleotides, or 200 nucleotides of megalomicin biosynthetic nucleic acid sequence, or a full-length megalomicin biosynthetic coding sequence. In another embodiment, the nucleic acids are smaller than 35, 200, or 500 nucleotides in length. Nucleic acids can be single or double stranded. Nucleic acids that hybridize to or are complementary to the foregoing sequences, in particular the inverse complement to nucleic acids that hybridize to the foregoing sequences (*i.e.*, the inverse complement of a nucleic acid strand has the complementary sequence running in reverse orientation to the strand so that the inverse complement would hybridize without mismatches to the nucleic acid strand) are also provided. In specific aspects, nucleic acids are provided which comprise a sequence complementary to (specifically are the inverse complement of) at least 10, 25, 50, 100, or 200 nucleotides or the entire coding region of a megalomicin biosynthetic gene.

The megalomicin biosynthetic nucleic acids provided herein include those with nucleotide sequences encoding substantially the same amino acid sequences as found in native megalomicin biosynthetic enzyme proteins, and those encoding amino acid sequences with functionally equivalent amino acids, as well as megalomicin biosynthetic enzyme derivatives or analogs as described in Section IV.

Some regions within the megalomicin PKS genes are highly homologous or identical to one another, as can be readily identified by an analysis of the sequence. The coding sequence for the KS and AT domains of module 2 shares significant identity with the coding sequence for the KS and AT domains of module 6. This sequence homology or identity at the nucleic acid, *e.g.*, DNA, level can render the nucleic acid unstable in certain host cells. To improve the stability

of the nucleic acids comprising a portion or the entire megalomicin PKS genes and megalomicin modification enzyme genes, the nucleic acid or DNA sequences can be changed to reduce or abolish the sequence homology or identity. Preferably, the DNA codons of homologous regions within the PKS or the megalomicin modification enzyme coding sequence are changed to reduce or abolish the sequence homology or identity without changing the amino acid sequences encoded by said changed DNA codons (see the examples below). The stability of the nucleic acid or DNA can also be improved by codon changes that reduce or abolish the sequence homology or identity while also changing the amino acid sequence, provided that the amino acid sequence change(s) does not substantially change the desired activity of the encoded megalomicin PKS. Thus, for example, one can simply substitute for the *megAIII* ORF an ORF from *eryAIII*, *oleAIII*, *picAIII*, or *picAIV* genes.

The recombinant DNA compounds of the invention that encode the megalomicin PKS and modification proteins or portions thereof are useful in a variety of applications. While many of these applications relate to the heterologous expression of the megalomicin biosynthetic genes or the construction of hybrid PKS enzymes, many useful applications involve the natural megalomicin producer *Micromonospora megalomicea*. For example, one can use the recombinant DNA compounds of the invention to disrupt the megalomicin biosynthetic genes by homologous recombination in *Micromonospora megalomicea*. The resulting host cell is a preferred host cell for making polyketides modified by oxidation, hydroxylation, glycosylation, and acylation in a manner similar to megalomicin, because the genes that encode the proteins that perform these reactions are of course present in the host cell, and because the host cell does not produce megalomicin that could interfere with production or purification of the polyketide of interest.

One illustrative recombinant host cell provided by the present invention expresses a recombinant megalomicin PKS in which the module 1 KS domain is inactivated by deletion or other mutation. In a preferred embodiment, the inactivation is mediated by a change in the KS domain that renders it incapable of binding substrate (called a KS1° mutation). In a particularly preferred embodiment, this inactivation is rendered by a mutation in the codon for the active

site cysteine that changes the codon to another codon, such as an alanine codon. Such constructs are especially useful when placed in translational reading frame with extender modules 1 and 2 of a megalomicin or the corresponding modules of another PKS. The utility of these constructs is that host cells expressing, or cell free extracts containing, a PKS comprising the protein encoded thereby can be fed or supplied with N-acylcysteamine thioesters of precursor molecules to prepare a polyketide of interest. See U.S. patent application Serial No. 09/492,773, filed 27 Jan. 2000, and PCT patent publication No. 00/44717, both of which are incorporated herein by reference. Such KS1° constructs of the invention are useful in the production of 13-substituted-megalomicin compounds in *Micromonospora megalomicea* host cells. Preferred compounds of the invention include those compounds in which the substituent at the 13-position is propyl, vinyl, propargyl, other lower alkyl, and substituted alkyl.

In a variant of this embodiment, one can employ a megalomicin PKS in which the ACP domain of module 1 has been rendered inactive. In another embodiment, one can delete the loading domain of the megalomicin PKS and provide monoketide substrates for processing by the remainder of the PKS.

The compounds of the invention can also be used to construct recombinant host cells of the invention in which coding sequences for one or more domains or modules of the megalomicin PKS or for another megalomicin biosynthetic gene have been deleted by homologous recombination with the *Micromonospora megalomicea* chromosomal DNA. Those of skill in the art will appreciate that the compounds used in the recombination process are characterized by their homology with the chromosomal DNA and not by encoding a functional protein due to their intended function of deleting or otherwise altering portions of chromosomal DNA. For this and a variety of other applications, the compounds of the present invention include not only those DNA compounds that encode functional proteins but also those DNA compounds that are complementary or identical to any portion of the megalomicin biosynthetic genes.

Thus, the invention provides a variety of modified *Micromonospora megalomicea* host cells in which one or more of the megalomicin biosynthetic genes have been mutated or disrupted. Transformation systems for *M. megalomicea* have been described by Hasegawa *et al.*, 1991, *J. Bacteriol.*

173:7004-11; and Takada *et al.*, 1994, *J. Antibiot. 47*:1167-1170, both of which are incorporated herein by reference. These cells are especially useful when it is desired to replace the disrupted function with a gene product expressed by a recombinant DNA expression vector. While such expression vectors of the invention are described in more detail in the following Section, those of skill in the art will appreciate that the vectors have application to *M. megalomicea* as well. Such *M. megalomicea* host cells can be preferred host cells for expressing megalomicin derivatives of the invention. Particularly preferred host cells of this type include those in which the coding sequence for the loading module has been mutated or disrupted, those in which one or more of any of the PKS gene ORFs has been mutated or disrupted, and/or those in which the genes for one or more modification (glycosylation, acylation, hydroxylation) have been mutated or disrupted.

While the present invention provides many useful compounds having application to, and recombinant host cells derived from, *Micromonospora megalomicea*, many important applications of the present invention relate to the heterologous expression of all or a portion of the megalomicin biosynthetic genes in cells other than *M. megalomicea*, as described in Section V.

Section IV: The Megalomicin Biosynthetic Enzymes and Antibodies Recognizing such Enzymes

In another specific embodiment, the invention provides a substantially purified polypeptide, which is encoded by a nucleic acid fragment comprising a nucleotide sequence encoding a domain of megalomicin polyketide synthase (PKS) or a megalomicin modification enzyme. The polypeptide can comprise a single domain, multiple domains or a full-length megalomicin PKS or megalomicin modification enzyme. Functional fragments, analogs or derivatives of the megalomicin PKS or megalomicin modification enzyme polypeptides are also provided. Preferably, such fragments, analogs or derivatives can be recognized an antibody raised against a megalomicin PKS or megalomicin modification enzyme. Also preferably, such fragments, analogs or derivatives comprise an amino acid sequence that has at least 60% identity, more preferably at least 90% identity to their wild type counterparts.

An exemplary nucleotide sequence encoding, and the corresponding amino acid sequence of, a megalomicin biosynthetic enzyme is disclosed in SEQ ID NO:1. Homologs (*e.g.*, nucleic acids of the above-listed genes of species other than *Micromonospora megalomicea*) or other related sequences (*e.g.*, paralogs) can be obtained by low, moderate or high stringency hybridization with all or a portion of the particular sequence provided as a probe using methods well known in the art for nucleic acid hybridization and cloning (*e.g.*, as described in Section III) in accordance with the methods of the present invention.

The megalomicin biosynthetic enzyme proteins, or domains thereof, of the present invention can be obtained by methods well known in the art for protein purification and recombinant protein expression in accordance with the methods of the present invention. For recombinant expression of one or more of the proteins, the nucleic acid containing all or a portion of the nucleotide sequence encoding the protein can be inserted into an appropriate expression vector, *i.e.*, a vector that contains the necessary elements for the transcription and translation of the inserted protein coding sequence. Transcriptional and translational signals can be supplied by the native promoter for a megalomicin biosynthetic gene and/or flanking regions.

A variety of host-vector systems may be utilized to express the protein coding sequence. These include but are not limited to mammalian cell systems infected with virus (*e.g.* vaccinia virus, adenovirus, and the like); insect cell systems infected with virus (*e.g.* baculovirus); microorganisms such as yeast containing yeast vectors; or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their properties. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used.

In a specific embodiment, a vector is used that comprises a promoter operably linked to nucleic acid sequences encoding a megalomicin biosynthetic enzyme, or a domain, fragment, derivative or homolog, thereof, one or more origins of replication, and optionally, one or more selectable markers (*e.g.*, an antibiotic resistance gene).

Expression vectors containing the sequences of interest can be identified by three general approaches: (a) nucleic acid hybridization, (b) presence or

absence of "marker" gene function, and (c) expression of the inserted sequences. In the first approach, megalomicin biosynthetic nucleic acid sequences can be detected by nucleic acid hybridization to probes comprising sequences homologous and complementary to the inserted sequences. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" functions (*e.g.*, binding to an anti-megalomicin biosynthetic enzyme antibody, resistance to antibiotics, occlusion body formation in baculovirus, and the like) caused by insertion of the sequences of interest in the vector. For example, if a megalomicin biosynthetic gene, or portion thereof, is inserted within the marker gene sequence of the vector, recombinants containing the megalomicin biosynthetic gene fragment will be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying for the megalomicin biosynthetic gene products expressed by the recombinant vector. Such assays can be based, for example, on the physical or functional properties of the interacting species in *in vitro* assay systems, *e.g.*, megalomicin synthesis activity, immunoreactivity to antibodies specific for the protein.

Once recombinant megalomicin biosynthetic genes or nucleic acids are identified, several methods known in the art can be used to propagate them in accordance with the methods of the present invention. Once a suitable host system and growth conditions have been established, recombinant expression vectors can be propagated and amplified in quantity. As previously described, the expression vectors or derivatives which can be used include, but are not limited to: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus, yeast vectors; bacteriophage vectors such as lambda phage; and plasmid and cosmid vectors.

In addition, a host cell strain may be chosen that modulates the expression of the inserted sequences, or modifies or processes the expressed proteins in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus expression of the genetically-engineered megalomicin biosynthetic enzymes may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (*e.g.* glycosylation, phosphorylation, and

the like) of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein is achieved. For example, expression in a bacterial system can be used to produce an unglycosylated core protein, while expression in mammalian cells ensures "native" glycosylation of a heterologous protein. Furthermore, different vector/host expression systems may effect processing reactions to different extent.

In particular, megalomicin biosynthetic enzyme derivatives can be made by altering their sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as an megalomicin biosynthetic gene can be used in the practice of the present invention. These include but are not limited to nucleotide sequences comprising all or portions of megalomicin biosynthetic genes that are altered by the substitution of different codons that encode the amino acid residue within the sequence, thus producing a silent change. Likewise, the megalomicin biosynthetic enzyme derivatives of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of megalomicin biosynthetic enzymes, including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

In a specific embodiment of the invention, the nucleic acids encoding proteins and proteins consisting of or comprising a domain or a fragment of megalomicin biosynthetic enzyme consisting of at least 6 (continuous) amino

acids are provided. In other embodiments, the domain or fragment consists of at least 10, 20, 30, 40, or 50 amino acids of a megalomicin biosynthetic enzyme. In specific embodiments, such domains or fragments are not larger than 35, 100 or 200 amino acids. Derivatives or analogs of megalomicin biosynthetic enzyme include but are not limited to molecules comprising regions that are substantially homologous to megalomicin biosynthetic enzyme in various embodiments, at least 30%, 40%, 50%, 60%, 70%, 80%, 90% or 95% identity over an amino acid sequence of identical size or when compared to an aligned sequence in which the alignment is done by a computer homology program known in the art in accordance with the methods of the present invention or whose encoding nucleic acid is capable of hybridizing to a sequence encoding a megalomicin biosynthetic enzyme under stringent, moderately stringent, or nonstringent conditions.

The megalomicin biosynthetic enzyme domains, derivatives and analogs of the invention can be produced by various methods known in the art in accordance with the methods of the present invention. The manipulations which result in their production can occur at the gene or protein level. For example, the cloned megalomicin biosynthetic gene sequence can be modified by any of numerous strategies known in the art (Sambrook et al., 1990, *Molecular Cloning, A Laboratory Manual,* 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) in accordance with the methods of the present invention. The sequences can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*.

Additionally, the megalomicin biosynthetic enzyme-encoding nucleotide sequence can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy pre-existing ones, to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art can be used in accordance with the methods of the present invention, including but not limited to, chemical mutagenesis and *in vitro* site-directed mutagenesis (Hutchinson et al., *J. Biol. Chem.* 253:6551-6558 (1978)), use of TAB® linkers (Pharmacia), and the like.

Once a recombinant cell expressing a megalomicin biosynthetic enzyme protein, or a domain, fragment or derivative thereof, is identified, the individual gene product can be isolated and analyzed. This is achieved by assays based on the physical and/or functional properties of the protein, including, but not limited to, radioactive labeling of the product followed by analysis by gel electrophoresis, immunoassay, cross-linking to marker-labeled product, and the like.

The megalomicin biosynthetic enzyme proteins may be isolated and purified by standard methods known in the art or recombinant host cells expressing the complexes or proteins in accordance with the methods of the invention, including but not restricted to column chromatography (*e.g.*, ion exchange, affinity, gel exclusion, reversed-phase high pressure, fast protein liquid, and the like), differential centrifugation, differential solubility, or by any other standard technique used for the purification of proteins. Functional properties may be evaluated using any suitable assay known in the art in accordance with the methods of the present invention.

Alternatively, once a megalomicin biosynthetic enzyme or its domain or derivative is identified, the amino acid sequence of the protein can be deduced from the nucleotide sequence of the gene which encodes it. As a result, the protein or its domain or derivative can be synthesized by standard chemical methods known in the art in accordance with the methods of the present invention (see Hunkapiller et al, *Nature* 310:105-111 (1984)).

Manipulations of megalomicin biosynthetic enzymes may be made at the protein level. Included within the scope of the invention are megalomicin biosynthetic enzyme domains, derivatives or analogs or fragments, which are differentially modified during or after translation, *e.g.*, by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, and the like. Any of numerous chemical modifications may be carried out by known techniques, including but not limited to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, $NaBH_4$, acetylation, formylation, oxidation, reduction, metabolic synthesis in the presence of tunicamycin, and the like.

In specific embodiments, the megalomicin biosynthetic enzymes are modified to include a fluorescent label. In other specific embodiments, the megalomicin biosynthetic enzyme is modified to have a heterofunctional reagent, such heterofunctional reagents can be used to crosslink the members of the complex.

In addition, domains, analogs and derivatives of a megalomicin biosynthetic enzyme can be chemically synthesized. For example, a peptide corresponding to a portion of a megalomicin biosynthetic enzyme, which comprises the desired domain or which mediates the desired activity *in vitro* can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the megalomicin biosynthetic enzyme sequence. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, alpha-amino isobutyric acid, 4-aminobutyric acid, 2-aminobutyric acid, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionoic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, ß-alanine, fluoro-amino acids, designer amino acids such as ß-methyl amino acids, Ca-methyl amino acids, Na-methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

In cases where natural products are suspected of being mutant or are isolated from new species, the amino acid sequence of the megalomicin biosynthetic enzyme isolated from the natural source, as well as those expressed *in vitro*, or from synthesized expression vectors *in vivo* or *in vitro*, can be determined from analysis of the DNA sequence, or alternatively, by direct sequencing of the isolated protein. Such analysis may be performed by manual sequencing or through use of an automated amino acid sequenator.

The megalomicin biosynthetic enzyme proteins may also be analyzed by hydrophilicity analysis (Hopp and Woods, *Proc. Natl. Acad. Sci. USA* 78:3824-3828 (1981)). A hydrophilicity profile can be used to identify the hydrophobic and hydrophilic regions of the proteins, and help predict their orientation in designing substrates for experimental manipulation, such as in binding

experiments, antibody synthesis, and the like. Secondary structural analysis can also be done to identify regions of the megalomicin biosynthetic enzyme that assume specific structures (Chou and Fasman, *Biochemistry* 13:222-23 (1974)). Manipulation, translation, secondary structure prediction, hydrophilicity and hydrophobicity profiles, open reading frame prediction and plotting, and determination of sequence homologies, can be accomplished using computer software programs available in the art.

Other methods of structural analysis including but not limited to X-ray crystallography (Engstrom, *Biochem. Exp. Biol.* 11:7-13 (1974)), mass spectroscopy and gas chromatography (Methods in Protein Science, J. Wiley and Sons, New York, 1997), and computer modeling (Fletterick and Zoller, eds., 1986, Computer Graphics and Molecular Modeling, In: *Current Communications in Molecular Biology,* Cold Spring Harbor Laboratory, Cold Spring Harbor Press, New York) can also be employed.

The invention also provides an antibody, or a fragment or derivative thereof, which immuno-specifically binds to a domain of megalomicin polyketide synthase (PKS) or a megalomicin modification enzyme. In a specific embodiment, an antibody which immuno-specifically binds to a domain of the megalomicin biosynthetic enzyme encoded by a nucleic acid that hybridizes to a nucleic acid having the nucleotide sequence set forth in the SEQ. ID NO:1, or a fragment or derivative of said antibody containing the binding domain thereof is provided. Preferably, the antibody is a monoclonal antibody.

The megalomicin biosynthetic enzyme protein and domains, fragments, homologs and derivatives thereof may be used as immunogens to generate antibodies which immunospecifically bind such immunogens. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library.

Various procedures known in the art may be used for the production of polyclonal antibodies to a megalomicin biosynthetic enzyme protein of the invention, its domains, derivatives, fragments or analogs in accordance with the methods of the present invention.

For production of the antibody, various host animals can be immunized by injection with the native megalomicin biosynthetic enzyme protein or a synthetic

version, or a derivative of the foregoing, such as a cross-linked megalomicin biosynthetic enzyme. Such host animals include but are not limited to rabbits, mice, rats, and the like. Various adjuvants can be used to increase the immunological response, depending on the host species, and include but are not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, dinitrophenol, and potentially useful human adjuvants such as bacille Calmette-Guerin (BCG) and corynebacterium parvum.

For preparation of monoclonal antibodies directed towards a megalomicin biosynthetic enzyme or domains, derivatives, fragments or analogs thereof, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used. Such techniques include but are not restricted to the hybridoma technique originally developed by Kohler and Milstein (*Nature* 256:495-497 (1975)), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., *Immunology Today* 4:72 (1983)), and the EBV hybridoma technique to produce human monoclonal antibodies (Cole et al., *in Monoclonal Antibodies and Cancer Therapy,* Alan R. Liss, Inc., pp. 77-96 (1985)). In an additional embodiment, monoclonal antibodies can be produced in germ-free animals (WO89/12690). Human antibodies may be used and can be obtained by using human hybridomas (Cote et al., *Proc. Natl. Acad. Sci. USA* 80:2026-2030 (1983)) or by transforming human B cells with EBV virus *in vitro* (Cole et al., *in Monoclonal Antibodies and Cancer Therapy,* Alan R. Liss, Inc., pp. 77-96 (1985)). Techniques developed for the production of "chimeric antibodies" (Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984); Neuberger et al., *Nature* 312:604-608 (1984); Takeda et al., *Nature* 314:452-454 (1985)) by splicing the genes from a mouse antibody molecule specific for the megalomicin biosynthetic enzyme protein together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention.

Techniques described for the production of single chain antibodies (U.S. patent 4,946,778) can be adapted to produce megalomicin biosynthetic enzyme-specific single chain antibodies. An additional embodiment utilizes the techniques described for the construction of Fab expression libraries (Huse et al., *Science*

246:1275-1281 (1989)) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for megalomicin biosynthetic enzyme, or domains, derivatives, or analogs thereof. Non-human antibodies can be "humanized" by known methods (*see, e.g.*, U.S. Patent No. 5,225,539).

Antibody fragments that contain the idiotypes of a megalomicin biosynthetic enzyme can be generated by techniques known in the art in accordance with the methods of the present invention. For example, such fragments include but are not limited to: the F(ab')2 fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments that can be generated by reducing the disulfide bridges of the F(ab')2 fragment, the Fab fragments that can be generated by treating the antibody molecular with papain and a reducing agent, and Fv fragments.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art in accordance with the methods of the present invention, *e.g.*, ELISA (enzyme-linked immunosorbent assay). To select antibodies specific to a particular domain of the megalomicin biosynthetic enzyme, one may assay generated hybridomas for a product that binds to the fragment of a megalomicin biosynthetic enzyme that contains such a domain.

The foregoing antibodies can be used in methods known in the art relating to the localization and/or quantitation of megalomicin biosynthetic enzyme proteins, *e.g.*, for imaging these proteins or measuring levels thereof in samples, in accordance with the methods of the present invention.

Section V: Heterologous Expression of the Megalomicin Biosynthetic Genes

In one important embodiment, the invention provides methods for the heterologous expression of one or more of the megalomicin biosynthetic genes and recombinant DNA expression vectors useful in the method. For purposes of the invention, any host cell other than *Micromonospora megalomicea* is a heterologous host cell. Thus, included within the scope of the invention in addition to isolated nucleic acids encoding domains, modules, or proteins of the megalomicin PKS and modification enzymes, are recombinant expression vectors that include such nucleic acids. The term expression vector refers to a nucleic acid that can be introduced into a host cell or cell-free transcription and translation

system. An expression vector can be maintained permanently or transiently in a cell, whether as part of the chromosomal or other DNA in the cell or in any cellular compartment, such as a replicating vector in the cytoplasm. An expression vector also comprises a promoter that drives expression of an RNA, which typically is translated into a polypeptide in the cell or cell extract. For efficient translation of RNA into protein, the expression vector also typically contains a ribosome-binding site sequence positioned upstream of the start codon of the coding sequence of the gene to be expressed. Other elements, such as enhancers, secretion signal sequences, transcription termination sequences, and one or more marker genes by which host cells containing the vector can be identified and/or selected, may also be present in an expression vector. Selectable markers, i.e., genes that confer antibiotic resistance or sensitivity, are preferred and confer a selectable phenotype on transformed cells when the cells are grown in an appropriate selective medium.

The various components of an expression vector can vary widely, depending on the intended use of the vector and the host cell(s) in which the vector is intended to replicate or drive expression. Expression vector components suitable for the expression of genes and maintenance of vectors in *E. coli*, yeast, *Streptomyces*, and other commonly used cells are widely known and commercially available. For example, suitable promoters for inclusion in the expression vectors of the invention include those that function in eucaryotic or procaryotic host cells. Promoters can comprise regulatory sequences that allow for regulation of expression relative to the growth of the host cell or that cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus. For *E. coli* and certain other bacterial host cells, promoters derived from genes for biosynthetic enzymes, antibiotic-resistance conferring enzymes, and phage proteins can be used and include, for example, the galactose, lactose (*lac*), maltose, tryptophan (*trp*), beta-lactamase (*bla*), bacteriophage lambda PL, and T5 promoters. In addition, synthetic promoters, such as the *tac* promoter (U.S. Patent No. 4,551,433), can also be used.

Thus, recombinant expression vectors contain at least one expression system, which, in turn, is composed of at least a portion of the megalomicin PKS and/or other megalomicin biosynthetic gene coding sequences operably linked to a

promoter and optionally termination sequences that operate to effect expression of the coding sequence in compatible host cells. The host cells are modified by transformation with the recombinant DNA expression vectors of the invention to contain the expression system sequences either as extrachromosomal elements or integrated into the chromosome. The resulting host cells of the invention are useful in methods to produce PKS and post-PKS modification enzymes as well as polyketides and antibiotics and other useful compounds derived therefrom.

Preferred host cells for purposes of selecting vector components for expression vectors of the present invention include fungal host cells such as yeast and procaryotic host cells such as *E. coli* and *Streptomyces*, but mammalian host cells can also be used. In hosts such as yeasts, plants, or mammalian cells that ordinarily do not produce polyketides, it may be necessary to provide, also typically by recombinant means, suitable holo-ACP synthases to convert the recombinantly produced PKS to functionality. Provision of such enzymes is described, for example, in PCT publication Nos. WO 97/13845 and 98/27203, each of which is incorporated herein by reference. Particularly preferred host cells for purposes of the present invention are *Streptomyces* and *Saccharopolyspora* host cells, as discussed in greater detail below.

In a preferred embodiment, the expression vectors of the invention are used to construct a heterologous recombinant *Streptomyces* host cell that expresses a recombinant PKS of the invention. *Streptomyces* is a convenient host for expressing polyketides, because polyketides are naturally produced in certain *Streptomyces* species, and *Streptomyces* cells generally produce the precursors needed to form the desired polyketide. Those of skill in the art will recognize that, if a *Streptomyces* host cell produces any portion of a PKS enzyme or produces a polyketide modification enzyme, the recombinant vector need drive expression of only those genes constituting the remainder of the desired PKS enzyme or other polyketide-modifying enzymes. Thus, such a vector may comprise only a single ORF, with the desired remainder of the polypeptides constituting the PKS provided by the genes on the host cell chromosomal DNA.

If a *Streptomyces* or other host cell ordinarily produces polyketides, it may be desirable to modify the host so as to prevent the production of endogenous polyketides prior to its use to express a recombinant PKS of the invention. Such

modified hosts include *S. coelicolor* CH999 and similarly modified *S. lividans* described in U.S. Patent No. 5,672,491, and PCT publication Nos. WO 95/08548 and WO 96/40968, incorporated herein by reference. In such hosts, it may not be necessary to provide enzymatic activities for all of the desired post-translational modifications of the enzymes that make up the recombinantly produced PKS, because the host naturally expresses such enzymes. In particular, these hosts generally contain holo-ACP synthases that provide the phosphopantotheinyl residue needed for functionality of the PKS.

The invention provides a wide variety of expression vectors for use in *Streptomyces*. The replicating expression vectors of the present invention include, for example and without limitation, those that comprise an origin of replication from a low copy number vector, such as SCP2* (see Hopwood *et al.*, *Genetic Manipulation of Streptomyces: A Laboratory manual* (The John Innes Foundation, Norwich, U.K., 1985); Lydiate *et al.*, 1985, *Gene 35*: 223-235; and Kieser and Melton, 1988, *Gene 65*: 83-91, each of which is incorporated herein by reference), SLP1.2 (Thompson *et al.*, 1982, *Gene 20*: 51-62, incorporated herein by reference), and pSG5(ts) (Muth *et al.*, 1989, *Mol. Gen. Genet. 219*: 341-348, and Bierman *et al.*, 1992, *Gene 116*: 43-49, each of which is incorporated herein by reference), or a high copy number vector, such as pIJ101 and pJV1 (see Katz *et al.*, 1983, *J. Gen. Microbiol. 129*: 2703-2714; Vara *et al.*, 1989, *J. Bacteriol. 171*: 5782-5781; and Servin-Gonzalez, 1993, *Plasmid 30*: 131-140, each of which is incorporated herein by reference). For non-replicating and integrating vectors and generally for any vector, it is useful to include at least an *E. coli* origin of replication, such as from pUC, p1P, p1I, and pBR. For phage based vectors, the phage phiC31 and its derivative KC515 can be employed (see Hopwood *et al.*, *supra*). Also, plasmid pSET152, plasmid pSAM, plasmids pSE101 and pSE211, all of which integrate site-specifically in the chromosomal DNA of *S. lividans*, can be employed for purposes of the present invention.

The *Streptomyces* recombinant expression vectors of the invention typically comprise one or more selectable markers, including antibiotic resistance conferring genes selected from the group consisting of the *ermE* (confers resistance to erythromycin and lincomycin), *tsr* (confers resistance to thiostrepton), *aadA* (confers resistance to spectinomycin and streptomycin), *aacC4*

(confers resistance to apramycin, kanamycin, gentamicin, geneticin (G418), and neomycin), *hyg* (confers resistance to hygromycin), and *vph* (confers resistance to viomycin) resistance conferring genes. Alternatively, several polyketides are naturally colored, and this characteristic can provide a built-in marker for identifying cells.

Megalomicins are currently produced only by the relatively genetically intractable host *Micromonospora megalomicinea*. This bacteria has not been commonly used in the fermentation industry for the large-scale production of antibiotics, and methods for high level production of megalomicin and its analogs are needed. In contrast, the streptomycete bacteria have been widely used for almost 50 years and are excellent hosts for production of megalomicin and its analogs. *Streptomyces lividans* and *S. coelicolor* have been developed for the expression of heterologous PKS systems. These organisms can stably maintain cloned heterologous PKS genes, express them at high levels under controlled conditions, and modify the corresponding PKS proteins (e.g., phosphopantotheinylation) so that they are capable of production of the polyketide they encode. Furthermore, these hosts contain the necessary pathways to produce the substrates required for polyketide synthesis; e.g. propionyl-CoA and methylmalonyl-CoA. A wide variety of cloning and expression vectors are available for these hosts, as are methods for the introduction and stable maintenance of large segments of foreign DNA. Relative to *Micromonospora* spp., *S. lividans* and *S. coelicolor* grow well on a number of media and have been adapted for high level production of polyketides in fermentors. If production levels are low, a number of rational approaches are available to improve yield (see Hosted and Baltz, 1996, *Trends Biotechnol. 14*(7):245-50, incorporated herein by reference). Empirical methods to increase the titers of these macrolides, long since proven effective for numerous bacterial polyketides, can also be employed.

Preferred *Streptomyces* host cell/vector combinations of the invention include *S. coelicolor* CH999 and *S. lividans* K4-114 host cells, which have been modified so as not to produce the polyketide actinorhodin, and expression vectors derived from the pRM1 and pRM5 vectors, as described in U.S. Patent Nos. 5,830,750 and 6,022,731 and U.S. patent application Serial No. 09/181,833, filed 28 Oct. 1998, each of which is incorporated herein by reference. These vectors are

particularly preferred in that they contain promoters compatible with numerous and diverse *Streptomyces spp*. Particularly useful promoters for *Streptomyces* host cells include those from PKS gene clusters that result in the production of polyketides as secondary metabolites, including promoters from aromatic (Type II) PKS gene clusters. Examples of Type II PKS gene cluster promoters are *act* gene promoters and *tcm* gene promoters; an example of a Type I PKS gene cluster promoter are the promoters of the spiramycin PKS genes and DEBS genes. The present invention also provides the megalomicin biosynthetic gene promoters in recombinant form. These promoters can be used to drive expression of the megalomicin biosynthetic genes or any other coding sequence of interest in host cells in which the promoter functions, particularly *Micromonospora megalomicea* and generally any *Streptomyces* species.

As described above, particularly useful control sequences are those that alone or together with suitable regulatory systems activate expression during transition from growth to stationary phase in the vegetative mycelium. The promoter contained in the aforementioned plasmid pRM5, i.e., the *actI/actIII* promoter pair and the *actII-ORF4* activator gene, is particularly preferred. Other useful *Streptomyces* promoters include without limitation those from the *ermE* gene and the *melC1* gene, which act constitutively, and the *tipA* gene and the *merA* gene, which can be induced at any growth stage. In addition, the T7 RNA polymerase system has been transferred to *Streptomyces* and can be employed in the vectors and host cells of the invention. In this system, the coding sequence for the T7 RNA polymerase is inserted into a neutral site of the chromosome or in a vector under the control of the inducible *merA* promoter, and the gene of interest is placed under the control of the T7 promoter. As noted above, one or more activator genes can also be employed to enhance the activity of a promoter. Activator genes in addition to the *actII-ORF4* gene described above include *dnrI*, *redD*, and *ptpA* genes (see U.S. patent application Serial No. 09/181,833, supra).

To provide a preferred host cell and vector for purposes of the invention, the megalomicin biosynthetic genes are placed on a recombinant expression vector and transferred to the non-macrolide producing hosts *Streptomyces lividans* K4-114 and *S. coelicolor* CH999. Transformation of *S. lividans* K4-114 or *S. coelicolor* CH999 with this expression vector results in a strain which produces

detectable amounts of megalomicin as determined by analysis of extracts by LC/MS. As noted above, the present invention also provides recombinant DNA compounds in which the encoded megalomicin module 1 KS domain is inactivated (the KS1° mutation). The introduction into *Streptomyces lividans* or *S. coelicolor* of a recombinant expression vector of the invention that encodes a megalomicin PKS with a KS1° domain produces a host cell useful for making polyketides by a process known as diketide feeding. The resulting host cells can be fed or supplied with N-acylcysteamine thioesters of precursor molecules to prepare megalomicin derivatives. Such cells of the invention are especially useful in the production of 13-substituted-6-deoxyerythronolide B compounds in recombinant host cells. Preferred compounds of the invention include those compounds in which the substituent at the 13-position is propyl, vinyl, propargyl, other lower alkyl, and substituted alkyl. In a preferred embodiment, the meg PKS is produced from a recombinant construct in which the *megAIII* gene has been altered to abolish the regions of identical coding sequence it otherwise shares with the *megAI* gene, or a hybrid PKS is employed in which the *megAIII* gene product has been replaced by the *oleAIII* gene product. Recombinant oleAIII genes are described in, for example, PCT patent publication No. 00/026349 and U.S. patent application Serial No. 09/428,517, filed 28 Oct. 1999, both of which are incorporated herein by reference.

The recombinant host cells of the invention can express all of the megalomicin biosynthetic genes or only a subset of the same. For example, if only the genes for the megalomicin PKS are expressed in a host cell that otherwise does not produce polyketide modifying enzymes that can act on the polyketide produced, then the host cell produces unmodified polyketides, called macrolide aglycones. Such macrolide aglycones can be hydroxylated and glycosylated by adding them to the fermentation of a strain such as, for example, *Streptomyces antibioticus* or *Saccharopolyspora erythraea*, that contains the requisite modification enzymes.

There are a wide variety of diverse organisms that can modify macrolide aglycones to provide compounds with, or that can be readily modified to have, useful activities. For example, as shown in Figure 5, *Saccharopolyspora erythraea* can convert 6-dEB to a variety of useful compounds. The erythronolide 6-dEB is

converted by the *eryF* gene product to erythronolide B, which is, in turn, glycosylated by the *eryBV* gene product to obtain 3-O-mycarosylerythronolide B, which contains L-mycarose at C-3. The *eryCIII* gene product then converts this compound to erythromycin D by glycosylation with D-desosamine at C-5. Erythromycin D, therefore, differs from 6-dEB through glycosylation and by the addition of a hydroxyl group at C-6. Erythromycin D can be converted to erythromycin B in a reaction catalyzed by the *eryG* gene product by methylating the L-mycarose residue at C-3. Erythromcyin D is converted to erythromycin C by the addition of a hydroxyl group at C-12 in a reaction catalyzed by the *eryK* gene product. Erythromycin A is obtained from erythromycin C by methylation of the mycarose residue in a reaction catalyzed by the *eryG* gene product. The unmodified megalomicin compounds provided by the present invention, such as, for example, the 6-dEB or 6-dEB analogs, produced in *Streptomyces lividans*, can be provided to cultures of *S. erythraea* and converted to the corresponding derivatives of erythromycins A, B, C, and D in accordance with the procedure provided in the examples below. To ensure that only the desired compound is produced, one can use an *S. erythraea eryA* mutant that is unable to produce 6-dEB but can still carry out the desired conversions (Weber *et al.*, 1985, *J. Bacteriol. 164*(1): 425-433). Also, one can employ other mutant strains, such as *eryB*, *eryC*, *eryG*, and/or *eryK* mutants, or mutant strains having mutations in multiple genes, to accumulate a preferred compound. The conversion can also be carried out in large fermentors for commercial production.

Moreover, there are other useful organisms that can be employed to hydroxylate and/or glycosylate the compounds of the invention. As described above, the organisms can be mutants unable to produce the polyketide normally produced in that organism, the fermentation can be carried out on plates or in large fermentors, and the compounds produced can be chemically altered after fermentation. Thus, *Streptomyces venezuelae*, which produces picromycin, contains enzymes that can transfer a desosaminyl group to the C-5 hydroxyl and a hydroxyl group to the C-12 position. In addition, *S. venezuelae* contains a glucosylation activity that glucosylates the 2'-hydroxyl group of the desosamine sugar. This latter modification reduces antibiotic activity, but the glucosyl residue is removed by enzymatic action prior to release of the polyketide from the cell.

Another organism, *S. narbonensis*, contains the same modification enzymes as *S. venezuelae*, except the C-12 hydroxylase. Thus, the present invention provides the compounds produced by hydroxylation and glycosylation of the macrolide aglycones of the invention by action of the enzymes endogenous to *S. narbonensis* and *S. venezuelae*.

Other organisms suitable for making compounds of the invention include *Micromonospora megalomicea* (discussed above), *Streptomyces antibioticus*, *S. fradiae*, and *S. thermotolerans*. *S. antibioticus* produces oleandomycin and contains enzymes that hydroxylate the C-6 and C-12 positions, glycosylate the C-3 hydroxyl with oleandrose and the C-5 hydroxyl with desosamine, and form an epoxide at C-8-C-8a. *S. fradiae* contains enzymes that glycosylate the C-5 hydroxyl with mycaminose and then the 4'-hydroxyl of mycaminose with mycarose, forming a disaccharide. *S. thermotolerans* contains the same activities as *S. fradiae*, as well as acylation activities. Thus, the present invention provides the compounds produced by hydroxylation and glycosylation of the macrolide aglycones of the invention by action of the enzymes endogenous to *S. antibioticus*, *S. fradiae*, and *S. thermotolerans*.

The present invention also provides methods and genetic constructs for producing the glycosylated and/or hydroxylated compounds of the invention directly in the host cell of interest. Thus, the recombinant genes of the invention, which include recombinant *megAI*, *megAII*, and *megAIII* genes with one or more deletions and/or insertions, including replacements of a *megA* gene fragment with a gene fragment from a heterologous PKS gene (as discussed in the next Section), can be included on expression vectors suitable for expression of the encoded gene products in *Saccharopolyspora erythraea*, *Streptomyces antibioticus*, *S. venezuelae*, *S. narbonensis*, *Micromonospora megalomicea*, *S. fradiae*, and *S. thermotolerans*.

A number of erythromycin high-producing strains of *Saccharopolyspora erythraea* and *Streptomyces fradiae* have been developed, and in a preferred embodiment, the megalomicin PKS and/or other megalomicin biosynthetic genes are introduced into such strains (or erythromycin non-producing mutants thereof) to provide the corresponding modified megalomicin compounds in high yields. Those of skill in the art will appreciate that *S. erythraea* contains the desosamine

and mycarose biosynthetic and transfer genes as well as DEBS, which, as noted above, makes the same macrolide aglycone, 6-dEB, as the megalomicin PKS. *S. erythraea* does not make megosamine or its corresponding transferase gene, and does not contain the acylation gene of *Micromonospora megalomicea*. Finally, the *S. erythraea eryG* gene product converts mycarose to cladinose, which does not occur in *M. megalomicea*. Thus, the present invention provides a wide variety of *S. erythraea* recombinant host cells, including, for example, those that contain:

(i) wild-type erythromycin biosynthetic genes with recombinant megosamine biosynthetic and transfer genes, with and without megalomicin acylation genes;

(ii) wild-type erythromycin biosynthetic genes except *eryG*, with recombinant megosamine biosynthetic and transfer genes, with and without megalomicin acylation genes; and

(iii) as in (i) and (ii), except that the *eryA* genes are inactive or deleted and recombinant *megA* genes have been introduced.

The invention provides other *S. erythraea* strains as well, including those in which any one or more of the erythromycin biosynthetic genes have been deleted or otherwise rendered inactive and in which at least one megalomicin biosynthetic gene has been introduced.

For example, the present invention enables one to express the megosamine genes in a *Saccharopolyspora erythraea eryG* mutant in which the erythromycin C made by this mutant is converted to megalomicin A. Alternatively, one could use an erythromycin C high -producing strain of *S. erythraea* in biotransformation methods in which the erythromycin C is fed to a *Streptomyces lividans* strain carrying only the megosamine biosynthesis and glycosyltransferase genes. As another alternative, one could use a strain of *S. lividans* that carries suitable erythromycin production genes along with the daunosamine biosynthesis genes plus *geneX* and *geneY* of Figure 5, or all of the megosamine biosynthesis genes, to produce megalomicin A.

All or some of the megalomicin gene cluster can be easily cloned under control of a suitable promoter in pCK7 or pSET152 either in one or two plasmids and introduced into the *Saccharopolyspora erythraea eryG* mutant. The *actII-ORF4/actI*p system and the phiC31/*int* system in pSET function well in this

organism (see Rowe *et al.*, 1998, *Gene*, 216:215-23, incorporated herein by reference). Alternatively, the megosamine biosynthesis genes are introduced into *Streptomyces lividans* on the same plasmids and the production of megalomicin A or its precursor mediated by bioconversion, done by feeding erythronolide B, 3-alpha-mycarosylerythronolide B, erythromycin D or erythromycin C to the *S. lividans* strain.

Lack of adequate resistance to megalomicin A in *S. erythraea* or *S. lividans* is not expected, because both organisms have MLS resistance genes (*ermE* and *mgt/lrm*, respectively), which confer resistance to several 14-membered macrolides (see Cundliffe, 1989, *Annu. Rev. Microbiol. 43*:207-33; Jenkins and Cundliffe, 1991, *Gene 108*:55-62; and Cundliffe, 1992, *Gene, 115*:75-84, each of which is incorporated herein by reference). One can also readily determine the level of resistance of the *S. erythraea eryG* mutant and the *S. lividans* host cells to megalomicin A, both in plate tests and in liquid medium. One can repeat the bioconversion method using an *eryG* mutant of a high erythromycin A producing *S. erythraea* strain (or an *eryB* or *eryC* mutant, as necessary) to determine the level at which megalomicin A can be produced. Furthermore, if experience shows that high level megalomicin A production requires a higher level of resistance to this macrolide than present in *S. erythraea* or *S. lividans*, the necessary megalomicin self-resistance genes will be cloned from *M. megalomicea* and moved into either one of the heterologous hosts. This will be straightforward work since self-resistance genes are usually found in the cluster of macrolide biosynthesis genes and can be identified by their homology to known macrolide resistance genes and(or) by the resistance phenotype they impart to a strain that normally is sensitive.

Alternatively, *geneX* and *geneY* (Figure 5) can be added to cassettes containing the relevant daunosamine (*dnm*) biosynthesis genes (Figure 5) to provide the ability to make TDP-megosamine *in vivo* and attach it to an erythromycin algycone. The TDP-daunosamine biosynthesis genes can be re-cloned from *Streptomyces peucetius* on two compatible and mutually selectable plasmids. When an *S. lividans* strain containing these two plasmids and the *dnmS* gene for TDP-daunosamine glycosyltransferase is grown in the presence of added epsilon-rhodomycinone, its glycoside with L-daunosamine, called rhodomycin D,

is produced in good yield. Thus, bioconversion of one of the erythromycins to megalomicin A should be observed when *geneX* and *geneY* are present. One can construct all five combination - the two *N*-dimethyltransferase genes and the three glycosyltransferase genes - to discriminate *geneX* and *geneY* from those connected with mycarose and desosamine biosynthesis and attachment in the megalomicin pathway.

Because the timing of megosamine addition is unknown, one can test erythronolide B, 3-alpha-mycarosylerythronolide B, erythromycin D and erythromycin C as substrates provided to a strain that expresses the megosamine biosynthetic and transferase genes. There is need to test the C3''' and(or) C4''' acylated metabolites like megalomicin C1, because these metabolites are made from megalomicin A and not the converse, based on the precedents in the biosynthesis of tylosin (see Arisawa *et al.*, 1994, *Appl. Environ. Microbiol. 60*: 2657-2661), carbomycin (see Epp *et al.*, 1989, *Gene 85*:293-301), and midecamycin (see Hara and Hutchinson, 1992, *J. Bacteriol. 174*, 5141-5144). If C-6 glycosylation of erythronolide B or 3-alpha-mycarosylerythronolide B (Figure 5) happens before addition of desosamine to C-5, then the erythromycin genes might not be able to complete formation of megalomicin A from some mono or diglycoside if the erythromycin glycosyltransferases cannot tolerate a C-6 glycoside. Although unexpected, such an outcome could be circumvented in accordance with the methods of the invention by cloning further megalomicin biosynthesis genes into the appropriate *S. erythraea* background or into *S. lividans* – specifically, the necessary deoxysugar biosynthesis and attachment genes – to create a recombinant strain that produces megalomicin A.

The acyltransferase gene that adds acetate or propionate to the C3''' or C4''' positions of mycarose in megalomicin B, C1 and C2 (Figure 3) is contained within the cosmids of the invention and can be identified by scanning the sequence data for the megalomicin gene cluster to locate homologs of *carE* and *mdmB* or their *acyA* homologs from the tylosin producer. The *carE* and *acyA* genes govern C4''' acylation in the carbomycin and tylosin pathway, respectively. The megalomicin homolog has the equivalent function in megalomicin biosynthesis (but is specific for C3''' and C4''' acylation). The gene can be cloned under control of a suitable promoter and introduced into *S. lividans* to produce the

desired acyl derivative of megalomicin A. Alternatively, introduction of the *carE* gene can form megalomicin B. This gene can be cloned from the carbomycin, spiramycin or tylosin producers.

If the amount of megalomicin produced by an *S. erythraea* or *S. lividans* or other recombinant host cell is less than desired, yield can be improved by optimizing the growth medium and fermentation conditions, by increasing expression of the gene(s) that appear to be rate limiting, based on the level of pathway intermediates that are accumulated by the recombinant strain constructed, and by reconstructing the *ery, dnm*, and megalomicin biosynthesis genes on vectors like pSET152 that can be integrated into the genome to provide a stabler recombinant strain for strain improvement.

In another embodiment, the present invention provides recombinant vectors encoding one or more of the megosamine, desosamine, and mycarose biosynthetic and transfer genes and heterologous host cells comprising those vectors. In this embodiment of the invention, the heterologous host cell is typically a cell that is unable to produce the sugar and transfer it to a polyketide unless the vector of the invention is introduced. For example, neither *Streptomyces lividans* nor *S. coelicolor* is naturally capable of making megosamine, desosamine, or mycarose or transferring those moieties to a polyketide. However, the present invention provides recombinant *Streptomyces lividans* and *S. coelicolor* host cells that are capable of making megosamine, desosamine, and/or mycarose and transferring those moieties to a polyketide.

Moreover, additional recombinant gene products can be expressed in the host cell to improve production of a desired polyketide. As but one non-limiting example, certain of the recombinant PKS proteins of the invention may produce a polyketide other than or in addition to the predicted polyketide, because the polyketide is cleaved from the PKS by the thioesterase (TE) domain in module 6 prior to processing by other domains on the PKS, in particular, any KR, DH, and/or ER domains in module 6. The production of the predicted polyketide can be increased in such instances by deleting the TE domain coding sequences from the gene and, optionally, expressing the TE domain as a separate protein. See Gokhale *et al.*, Feb. 1999, "Mechanism and specificity of the terminal thioesterase

domain from the erythromycin polyketide synthase," *Chem. & Biol. 6*: 117-125, incorporated herein by reference.

Thus, in one important aspect, the present invention provides methods, expression vectors, and recombinant host cells that enable the production of megalomicin and hydroxylated and glycosylated derivatives of megalomicin in heterologous host cells. The present invention also provides methods for making a wide variety of polyketides derived in part from the megalomicin PKS or other biosynthetic genes, as described in the following Section.

Section VI: Hybrid PKS Genes

The present invention provides recombinant DNA compounds encoding each of the domains of each of the modules of the megalomicin PKS as well as the other megalomicin biosynthetic enzymes. The availability of these compounds permits their use in recombinant procedures for production of desired portions of the megalomicin PKS fused to or expressed in conjunction with all or a portion of a heterologous PKS and, optionally, one or more polyketide modification enzymes. These compounds also permit the modification of polyketides with the various megalomicin modification enzymes. The resulting hybrid PKS can then be expressed in a host cell to produce a desired polyketide or modified form thereof.

Thus, in accordance with the methods of the invention, a portion of the megalomicin biosynthetic gene coding sequence that encodes a particular activity can be isolated and manipulated, for example, to replace the corresponding region in a different modular PKS gene or modification enzyme gene. In addition, coding sequences for individual proteins, modules, domains, and portions thereof of the megalomicin PKS can be ligated into suitable expression systems and used to produce the portion of the protein encoded. The resulting protein can be isolated and purified or can may be employed *in situ* to effect polyketide synthesis. Depending on the host for the recombinant production of the domain, module, protein, or combination of proteins, suitable control sequences such as promoters, termination sequences, enhancers, and the like are ligated to the nucleotide sequence encoding the desired protein in the construction of the expression vector, as described above.

In one important embodiment, the invention thus provides hybrid PKS enzymes and the corresponding recombinant DNA compounds that encode those hybrid PKS enzymes. For purposes of the invention, a hybrid PKS is a recombinant PKS that comprises all or part of one or more extender modules, loading module, and/or thioesterase/cyclase domain of a first PKS and all or part of one or more extender modules, loading module, and/or thioesterase/cyclase domain of a second PKS. In one preferred embodiment, the first PKS is most but not all of the megalomicin PKS, and the second PKS is only a portion of a non-megalomicin PKS. An illustrative example of such a hybrid PKS includes a megalomicin PKS in which the megalomicin PKS loading module has been replaced with a loading module of another PKS. Another example of such a hybrid PKS is a megalomicin PKS in which the AT domain of extender module 3 is replaced with an AT domain that binds only malonyl CoA. In another preferred embodiment, the first PKS is most but not all of a non-megalomicin PKS, and the second PKS is only a portion of the megalomicin PKS. An illustrative example of such a hybrid PKS includes a rapamycin PKS in which an AT specific for malonyl CoA is replaced with the AT from the megalomicin PKS specific for methylmalonyl CoA. Other illustrative hybrid PKSs of the invention are described below.

Those of skill in the art will recognize that all or part of either the first or second PKS in a hybrid PKS of the invention need not be isolated from a naturally occurring source. For example, only a small portion of an AT domain determines its specificity. See PCT patent application No. WO US99/15047, and Lau *et al.*, *infra*, incorporated herein by reference. The state of the art in DNA synthesis allows the artisan to construct *de novo* DNA compounds of size sufficient to construct a useful portion of a PKS module or domain. Thus, the desired derivative coding sequences can be synthesized using standard solid phase synthesis methods such as those described by Jaye *et al.*, 1984, *J. Biol. Chem. 259*: 6331, and instruments for automated synthesis are available commercially from, for example, Applied Biosystems, Inc. For purposes of the invention, such synthetic DNA compounds are deemed to be a portion of a PKS.

With this general background regarding hybrid PKSs of the invention, one can better appreciate the benefit provided by the DNA compounds of the invention

that encode the individual domains, modules, and proteins that comprise the megalomicin PKS. As described above, the megalomicin PKS is comprised of a loading module, six extender modules composed of a KS, AT, ACP, and zero, one, two, or three KR, DH, and ER domains, and a thioesterase domain. The DNA compounds of the invention that encode these domains individually or in combination are useful in the construction of the hybrid PKS encoding DNA compounds of the invention. For example, a DNA compound of the invention that encodes an extender module or portion of an extender module is useful in the construction of a coding sequence that encodes a protein subcomponent of a PKS. The DNA compound of the invention that comprises a coding sequence of a PKS subunit protein is useful in the construction of an expression vector that drives expression of the subunit in a host cell that expresses the other subunits and so produces a functional PKS.

The recombinant DNA compounds of the invention that encode the loading module of the megalomicin PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the megalomicin PKS loading module is inserted into a DNA compound that comprises the coding sequence for one or more heterologous PKS extender modules. The resulting construct, in which the coding sequence for the loading module of the heterologous PKS is replaced by that for the coding sequence of the megalomicin PKS loading module provides a novel PKS. Examples include the DEBS, rapamycin, FK-506, FK-520, rifamycin, and avermectin PKS coding sequences. In another embodiment, a DNA compound comprising a sequence that encodes the megalomicin PKS loading module is inserted into a DNA compound that comprises the coding sequence for the megalomicin PKS or a recombinant megalomicin PKS that produces a megalomicin derivative.

In another embodiment, a portion of the loading module coding sequence is utilized in conjuction with a heterologous coding sequence. In this embodiment, the invention provides, for example, replacing the methylmalonyl CoA (propionyl) specific AT with a malonyl CoA (acetyl), ethylmalonyl CoA (butyryl), or other CoA specific AT. In addition, the AT and/or ACP can be replaced by another AT and/or another ACP or an inactivated KS, such as a KS^Q, an AT, and/or another

ACP. The resulting heterologous loading module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes megalomicin, a megalomicin derivative, or another polyketide.

The recombinant DNA compounds of the invention that encode the first extender module of the megalomicin PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the megalomicin PKS first extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the first extender module of the megalomicin PKS or the latter is merely added to coding sequences for modules of the heterologous PKS, provides a novel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encodes the first extender module of the megalomicin PKS is inserted into a DNA compound that comprises coding sequences for the megalomicin PKS or a recombinant megalomicin PKS that produces a megalomicin derivative.

In another embodiment, a portion or all of the first extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting (which includes inactivating) the KR; inserting a DH or a DH and ER; and/or replacing the KR with another KR, a DH and KR, or a DH, KR, and ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the megalomicin PKS, from a gene for a PKS that produces a polyketide other than megalomicin, or from chemical synthesis. The resulting heterologous first extender module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes megalomicin, a megalomicin derivative, or another polyketide.

Those of skill in the art will recognize, however, that deletion of the KR domain of extender module 1 or insertion of a DH domain or DH and KR domains

into extender module 1 will prevent the typical cyclization of the polyketide at the hydroxyl group created by the KR if such hybrid module is employed as a first extender module in a hybrid PKS or is otherwise involved in producing a portion of the polyketide at which cyclization is to occur. Such deletions or insertions can be useful, however, to create linear molecules or to induce cyclization at another site in the molecule.

As noted above, the invention also provides recombinant PKSs and recombinant DNA compounds and vectors that encode such PKSs in which the KS domain of the first extender module has been inactivated. Such constructs are typically expressed in translational reading frame with the first two extender modules on a single protein, with the remaining modules and domains of a megalomicin, megalomicin derivative, or hybrid PKS expressed as one or more, typically two, proteins to form the multi-protein functional PKS. The utility of these constructs is that host cells expressing, or cell free extracts containing, the PKS encoded thereby can be fed or supplied with N-acylcysteamine thioesters of precursor molecules to prepare megalomicin derivative compounds. See U.S. patent application Serial No. 09/492,733, filed 27 Jan. 2000, and PCT publication Nos. WO 00/44717, 99/03986 and 97/02358, each of which is incorporated herein by reference.

The recombinant DNA compounds of the invention that encode the second extender module of the megalomicin PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the megalomicin PKS second extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the second extender module of the megalomicin PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS. In another embodiment, a DNA compound comprising a sequence that encodes the second extender module of the megalomicin PKS is inserted into a DNA compound that comprises the coding sequences for the megalomicin PKS or a recombinant megalomicin PKS that produces a megalomicin derivative.

In another embodiment, a portion or all of the second extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting (or inactivating) the KR; replacing the KR with a KR, a KR and a DH, or a KR, DH, and ER; and/or inserting a DH or a DH and an ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the megalomicin PKS, from a coding sequence for a PKS that produces a polyketide other than megalomicin, or from chemical synthesis. The resulting heterologous second extender module coding sequence can be utilized in conjunction with a coding sequence from a PKS that synthesizes megalomicin, a megalomicin derivative, or another polyketide.

The recombinant DNA compounds of the invention that encode the third extender module of the megalomicin PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the megalomicin PKS third extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the third extender module of the megalomicin PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS. In another embodiment, a DNA compound comprising a sequence that encodes the third extender module of the megalomicin PKS is inserted into a DNA compound that comprises coding sequences for the megalomicin PKS or a recombinant megalomicin PKS that produces a megalomicin derivative.

In another embodiment, a portion or all of the third extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting the inactive KR; and/or

replacing the KR with an active KR, or a KR and DH, or a KR, DH, and ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the megalomicin PKS, from a gene for a PKS that produces a polyketide other than megalomicin, or from chemical synthesis. The resulting heterologous third extender module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes megalomicin, a megalomicin derivative, or another polyketide.

The recombinant DNA compounds of the invention that encode the fourth extender module of the megalomicin PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the megalomicin PKS fourth extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the fourth extender module of the megalomicin PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS. In another embodiment, a DNA compound comprising a sequence that encodes the fourth extender module of the megalomicin PKS is inserted into a DNA compound that comprises coding sequences for the megalomicin PKS or a recombinant megalomicin PKS that produces a megalomicin derivative.

In another embodiment, a portion of the fourth extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting or inactivating any one, two, or all three of the ER, DH, and KR; and/or replacing any one, two, or all three of the ER, DH, and KR with either a KR, a DH and KR, or a KR, DH, and ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the megalomicin PKS (except for the DH and ER domains), from a coding sequence

for a PKS that produces a polyketide other than megalomicin, or from chemical synthesis. The resulting heterologous fourth extender module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes megalomicin, a megalomicin derivative, or another polyketide.

The recombinant DNA compounds of the invention that encode the fifth extender module of the megalomicin PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the megalomicin PKS fifth extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the fifth extender module of the megalomicin PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS. In another embodiment, a DNA compound comprising a sequence that encodes the fifth extender module of the megalomicin PKS is inserted into a DNA compound that comprises the coding sequence for the megalomicin PKS or a recombinant megalomicin PKS that produces a megalomicin derivative.

In another embodiment, a portion or all of the fifth extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting (or inactivating) the KR; inserting a DH or a DH and ER; and/or replacing the KR with another KR, a DH and KR, or a DH, KR, and ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the megalomicin PKS, from a coding sequence for a PKS that produces a polyketide other than megalomicin, or from chemical synthesis. The resulting heterologous fifth extender module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes megalomicin, a megalomicin derivative, or another polyketide.

The recombinant DNA compounds of the invention that encode the sixth extender module of the megalomicin PKS and the corresponding polypeptides

encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the megalomicin PKS sixth extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the sixth extender module of the megalomicin PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS. In another embodiment, a DNA compound comprising a sequence that encodes the sixth extender module of the megalomicin PKS is inserted into a DNA compound that comprises the coding sequences for the megalomicin PKS or a recombinant megalomicin PKS that produces a megalomicin derivative.

In another embodiment, a portion or all of the sixth extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting or inactivating the KR or replacing the KR with another KR, a KR and DH, or a KR, DH, and an ER; and/or inserting a DH or a DH and ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the megalomicin PKS, from a coding sequence for a PKS that produces a polyketide other than megalomicin, or from chemical synthesis. The resulting heterologous sixth extender module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes megalomicin, a megalomicin derivative, or another polyketide.

The sixth extender module of the megalomicin PKS is followed by a thioesterase domain. This domain is important in the cyclization of the polyketide and its cleavage from the PKS. The present invention provides recombinant DNA compounds that encode hybrid PKS enzymes in which the megalomicin PKS is fused to a heterologous thioesterase or a heterologous PKS is fused to the megalomicin PKS thioesterase. Thus, for example, a thioesterase domain coding sequence from another PKS gene can be inserted at the end of the sixth (or other final) extender module coding sequence in recombinant DNA compounds of the

invention or the megalomicin PKS thioesterase can be similarly fused to a heterologous PKS. Recombinant DNA compounds encoding this thioesterase domain are useful in constructing DNA compounds that encode the megalomicin PKS, a PKS that produces a megalomicin derivative, and a PKS that produces a polyketide other than megalomicin or a megalomicin derivative.

Thus, the hybrid modules of the invention are incorporated into a PKS to provide a hybrid PKS of the invention. A hybrid PKS of the invention can result not only:

(i) from fusions of heterologous domain (where heterologous means the domains in a module are derived from at least two different naturally occurring modules) coding sequences to produce a hybrid module coding sequence contained in a PKS gene whose product is incorporated into a PKS,

but also:

(ii) from fusions of heterologous modules (where heterologous module means two modules are adjacent to one another that are not adjacent to one another in naturally occurring PKS enzymes) coding sequences to produce a hybrid coding sequence contained in a PKS gene whose product is incorporated into a PKS,

(iii) from expression of one or more megalomicin PKS genes with one or more non-megalomicin PKS genes, including both naturally occurring and recombinant non-megalomicin PKS genes, and

(iv) from combinations of the foregoing.

Various hybrid PKSs of the invention illustrating these various alternatives are described herein.

An example of a hybrid PKS comprising fused modules results from fusion of the loading module of either the DEBS PKS or the narbonolide PKS (see PCT patent application No. US99/11814, incorporated herein by reference) with extender modules 1 and 2 of the megalomicin PKS to produce a hybrid *megAI* gene. Co-expression of either one of these two hybrid *megAI* genes with the *megAII* and *megAIII* genes in suitable host cells, such as *Streptomcyes lividans*, results in expression of a hybrid PKS of the invention that produces 6-deoxyerythronolide B (the polyketide product of the natural *megA* genes) in recombinant host cells. Co-expression of either one of these two hybrid *megAI*

genes with the *eryAII* and *eryAIII* genes similarly results in the production of 6-dEB, while co-expression with the analogous narbonolide PKS genes, *picAII*, *picAIII* and *picAIV*, results in the production of 3-deoxy-3-oxo-6-dEB (3-keto-6-dEB), useful in the production of ketolides, compounds with potent anti-bacterial activity.

Another example of a hybrid PKS comprising a hybrid module is prepared by co-expressing the *megAI* and *megAII* genes with a *megAIII* hybrid gene encoding extender module 5 and the KS and AT of extender module 6 of the megalomicin PKS fused to the ACP of module 6 and the TE of the narbonolide PKS. The resulting hybrid PKS of the invention produces 3-keto-6-dEB. This compound can also be prepared by a recombinant megalomicin derivative PKS of the invention in which the KR domain of module 6 of the megalomicin PKS has been deleted. Moreover, the invention provides hybrid PKSs in which not only the above changes have been made but also the AT domain of module 6 has been replaced with a malonyl-specific AT. These hybrid PKSs produce 2-desmethyl-3-deoxy-3-oxo-6-dEB, a useful intermediate in the preparation of 2-desmethyl ketolides, compounds with potent antibiotic activity.

Another illustrative example of a hybrid PKS includes the hybrid PKS of the invention resulting only from the latter change in the hybrid PKS just described. Thus, co-expression of the *megAI* and *megAII* genes with a hybrid *megAIII* gene in which the AT domain of module 6 has been replaced by a malonyl-specific AT results in the expression of a hybrid PKS that produces 2-desmethyl-6-dEB in recombinant host cells. This compound is a useful intermediate for making 2-desmethyl erythromycins in recombinant host cells of the invention, as well as for making 2-desmethyl semi-synthetic ketolides.

While many of the hybrid PKSs described above are composed primarily of megalomicin PKS proteins, those of skill in the art recognize that the present invention provides many different hybrid PKSs, including those composed of only a small portion of the megalomicin PKS. For example, the present invention provides a hybrid PKS in which a hybrid *eryAI* gene that encodes the megalomicin PKS loading module fused to extender modules 1 and 2 of DEBS is coexpressed with the *eryAII* and *eryAIII* genes. The resulting hybrid PKS produces 6-dEB, the product of the native DEBS. When the construct is expressed in

Saccharopolyspora erythraea host cells (either via chromosomal integration in the chromosome or via a vector that encodes the hybrid PKS), the resulting recombinant host cell of the invention produces erythromycins. Another illustrative example is the hybrid PKS of the invention composed of the *megAI* and *eryAII* and *eryAIII* gene products. This construct is also useful in expressing erythromycins in *Saccharopolyspora erythraea* host cells. In a preferred embodiment, the *S. erythraea* host cells are *eryAI* mutants that do not produce 6-deoxyerythronolide B.

Another example is the hybrid PKS of the invention composed of the products of the *picAI* and *picAII* genes (the two proteins that comprise the loading module and extender modules 1 - 4, inclusive, of the narbonolide PKS) and the *megAIII* gene. The resulting hybrid PKS produces the macrolide aglycone 3-hydroxy-narbonolide in *Streptomyces lividans* host cells and the corresponding erythromycins in *Saccharopolyspora erythraea* host cells.

Each of the foregoing hybrid PKS enzymes of the invention, and the hybrid PKS enzymes of the invention generally, can be expressed in a host cell that also expresses a functional *oleP* gene product. The *oleP* gene encodes an oleandomycin modification enzyme, and expression of the gene together with a hybrid PKS of the invention provides the compounds of the invention in which a C-8 hydroxyl, a C-8a or C-8-C-8a epoxide is present.

Recombinant methods for manipulating modular PKS genes to make hybrid PKS enzymes are described in U.S. Patent Nos. 5,672,491; 5,843,718; 5,830,750; and 5,712,146; and in PCT publication Nos. 98/49315 and 97/02358, each of which is incorporated herein by reference. A number of genetic engineering strategies have been used with DEBS to demonstrate that the structures of polyketides can be manipulated to produce novel natural products, primarily analogs of the erythromycins (see the patent publications referenced *supra* and Hutchinson, 1998, *Curr Opin Microbiol. 1*:319-329, and Baltz, 1998, *Trends Microbiol. 6*:76-83, incorporated herein by reference). Because of the similar activity of the megalomicin PKS and DEBS (both PKS enzymes produce the macrolide aglycone 6-dEB), these methods can be readily applied to the recombinant megalomicin PKS genes of the invention.

These techniques include: (i) deletion or insertion of modules to control chain length, (ii) inactivation of reduction/dehydration domains to bypass beta-carbon processing steps, (iii) substitution of AT domains to alter starter and extender units, (iv) addition of reduction/dehydration domains to introduce catalytic activities, and (v) substitution of ketoreductase KR domains to control hydroxyl stereochemistry. In addition, engineered blocked mutants of DEBS have been used for precursor directed biosynthesis of analogs that incorporate synthetically derived starter units. For example, more than 100 novel polyketides were produced by engineering single and combinatorial changes in multiple modules of DEBS. Hybrid PKS enzymes based on DEBS with up to three catalytic domain substitutions were constructed by cassette mutagenesis, in which various DEBS domains were replaced with domains from the rapamycin PKS (see Schweke *et al.*, 1995, *Proc. Nat. Acad. Sci. USA 92*, 7839-7843, incorporated herein by reference) or one more of the DEBS KR domains was deleted. Functional single domain replacements or deletions were combined to generate DEBS enzymes with double and triple catalytic domain substitutions (see McDaniel *et al.*, 1999, *Proc. Nat. Acad. Sci. USA 96*, 1846-1851, incorporated herein by reference). By providing the analogous megalomicin/rapamycin hybrid PKS enzymes, the present invention provides alternative means to make these polyketides.

Methods for generating libraries of polyketides have been greatly improved by cloning PKS genes as a set of three or more mutually selectable plasmids, each carrying a different wild-type or mutant PKS gene, then introducing all possible combinations of the plasmids with wild-type, mutant, and hybrid PKS coding sequences into the same host (see U.S. patent application Serial No. 60/129,731, filed 16 Apr. 1999, and PCT Pub. No. 98/27203, each of which is incorporated herein by reference). This method can also incorporate the use of a KS1° mutant, which by mutational biosynthesis can produce polyketides made from diketide starter units (see Jacobsen *et al.*, 1997, *Science* 277, 367-369, incorporated herein by reference), as well as the use of a truncated gene that leads to 12-membered macrolides or an elongated gene that leads to 16-membered ketolides. Moreover, by utilizing in addition one or more vectors that encode glycosyl biosynthesis and transfer genes, such as those of the present invention for megosamine,

desosamine, oleandrose, cladinose, and/or mycarose (in any combination), a large collection of glycosylated polyketides can be prepared.

The following Table lists references describing illustrative PKS genes and corresponding enzymes that can be utilized in the construction of the recombinant hybrid PKSs and the corresponding DNA compounds that encode them of the invention. Also presented are various references describing tailoring enzymes and corresponding genes that can be employed in accordance with the methods of the invention.

Avermectin

U.S. Pat. No. 5,252,474 to Merck.

MacNeil *et al.*, 1993, Industrial Microorganisms: Basic and Applied Molecular Genetics, Baltz, Hegeman, & Skatrud, eds. (ASM), pp. 245-256, A Comparison of the Genes Encoding the Polyketide Synthases for Avermectin, Erythromycin, and Nemadectin.

MacNeil *et al.*, 1992, *Gene 115*: 119-125, Complex Organization of the *Streptomyces avermitilis* genes encoding the avermectin polyketide synthase.

Candicidin (FR008)

Hu *et al.*, 1994, *Mol. Microbiol.* 14: 163-172.

Epothilone

PCT Pub. No. 00/031247 to Kosan.

Erythromycin

PCT Pub. No. 93/13663 to Abbott.

US Pat. No. 5,824,513 to Abbott.

Donadio *et al.*, 1991, *Science 252*:675-9.

Cortes *et al.*, 8 Nov. 1990, *Nature 348*:176-8, An unusually large multifunctional polypeptide in the erythromycin producing polyketide synthase of *Saccharopolyspora erythraea*.

Glycosylation Enzymes

PCT Pub. No. 97/23630 to Abbott.

FK-506

Motamedi *et al.*, 1998, The biosynthetic gene cluster for the macrolactone ring of the immunosuppressant FK506, *Eur. J. biochem. 256*: 528-534.

Motamedi *et al.*, 1997, Structural organization of a multifunctional polyketide synthase involved in the biosynthesis of the macrolide immunosuppressant FK506, *Eur. J. Biochem. 244*: 74-80.

Methyltransferase

US 5,264,355, issued 23 Nov. 1993, Methylating enzyme from *Streptomyces* MA6858. 31-O-desmethyl-FK506 methyltransferase.

Motamedi *et al.*, 1996, Characterization of methyltransferase and hydroxylase genes involved in the biosynthesis of the immunosuppressants FK506 and FK520, *J. Bacteriol. 178*: 5243-5248.

FK-520

PCT Pub. No. 00/20601 to Kosan.

See also Nielsen *et al.*, 1991, *Biochem. 30*:5789-96 (enzymology of pipecolate incorporation).

Lovastatin

U.S. Pat. No. 5,744,350 to Merck.

Narbomycin (and Picromycin)

PCT Pub. No. WO US99/61599 to Kosan.

Nemadectin

MacNeil *et al.*, 1993, *supra.*

Niddamycin

Kakavas *et al.*, 1997, Identification and characterization of the niddamycin polyketide synthase genes from *Streptomyces caelestis*, *J. Bacteriol. 179*: 7515-7522.

Oleandomycin

Swan *et al.*, 1994, Characterization of a *Streptomyces antibioticus* gene encoding a type I polyketide synthase which has an unusual coding sequence, *Mol. Gen. Genet. 242*: 358-362.

PCT Pub. No. 00/026349 to Kosan.

Olano *et al.*, 1998, Analysis of a *Streptomyces antibioticus* chromosomal region involved in oleandomycin biosynthesis, which encodes two glycosyltransferases responsible for glycosylation of the macrolactone ring, *Mol. Gen. Genet. 259*(3): 299-308.

Platenolide

EP Pub. No. 791,656 to Lilly.

Rapamycin

Schwecke *et al.*, Aug. 1995, The biosynthetic gene cluster for the polyketide rapamycin, *Proc. Natl. Acad. Sci. USA 92*:7839-7843.

Aparicio *et al.*, 1996, Organization of the biosynthetic gene cluster for rapamycin in *Streptomyces hygroscopicus*: analysis of the enzymatic domains in the modular polyketide synthase, *Gene 169*: 9-16.

Rifamycin

August *et al.*, 13 Feb. 1998, Biosynthesis of the ansamycin antibiotic rifamycin: deductions from the molecular analysis of the *rif* biosynthetic gene cluster of *Amycolatopsis mediterranei* S669, *Chemistry & Biology, 5*(2): 69-79.

Soraphen

U.S. Pat. No. 5,716,849 to Novartis.

Schupp *et al.*, 1995, *J. Bacteriology 177*: 3673-3679. A *Sorangium cellulosum* (Myxobacterium) Gene Cluster for the Biosynthesis of the Macrolide Antibiotic Soraphen A: Cloning, Characterization, and Homology to Polyketide Synthase Genes from Actinomycetes.

Spiramycin

U.S. Pat. No. 5,098,837 to Lilly.

Activator Gene

U.S. Pat. No. 5,514,544 to Lilly.

Tylosin

EP Pub. No. 791,655 to Lilly.

Kuhstoss *et al.*, 1996, *Gene 183*:231-6., Production of a novel polyketide through the construction of a hybrid polyketide synthase.

U.S. Pat. No. 5,876,991 to Lilly.

Tailoring enzymes

Merson-Davies and Cundliffe, 1994, *Mol. Microbiol. 13*: 349-355. Analysis of five tylosin biosynthetic genes from the *tylBA* region of the *Streptomyces fradiae* genome.

As the above Table illustrates, there are a wide variety of PKS genes that serve as readily available sources of DNA and sequence information for use in constructing the hybrid PKS-encoding DNA compounds of the invention.

In constructing hybrid PKSs of the invention, certain general methods may be helpful. For example, it is often beneficial to retain the framework of the module to be altered to make the hybrid PKS. Thus, if one desires to add DH and ER functionalities to a module, it is often preferred to replace the KR domain of the original module with a cognate KR, DH, and ER domain-containing segment from another module, instead of merely inserting DH and ER domains. One can alter the stereochemical specificity of a module by replacement of the KS domain with a KS domain from a module that specifies a different stereochemistry. See Lau *et al.*, 1999, "Dissecting the role of acyltransferase domains of modular polyketide synthases in the choice and stereochemical fate of extender units" *Biochemistry 38*(5):1643-1651, incorporated herein by reference. One can alter the specificity of an AT domain by changing only a small segment of the domain. See Lau *et al.*, *supra*. One can also take advantage of known linker regions in PKS proteins to link modules from two different PKSs to create a hybrid PKS. See Gokhale *et al.*, 16 Apr. 1999, Dissecting and Exploiting Intermodular Communication in Polyketide Synthases", *Science 284*: 482-485, incorporated herein by reference.

The hybrid PKS-encoding DNA compounds of the invention can be and often are hybrids of more than two PKS genes. Even where only two genes are used, there are often two or more modules in the hybrid gene in which all or part of the module is derived from a second (or third) PKS gene. Thus, as one illustrative example, the invention provides a hybrid PKS that contains the naturally occurring loading module and thioesterase domain as well as extender modules one, two, four, and six of the megalomicin PKS and further contains hybrid or heterologous extender modules three and five. Hybrid or heterologous extender modules three and five contain AT domains specific for malonyl CoA and derived from, for example, the rapamycin PKS genes.

The invention also provides libraries of PKS genes, PKS proteins, and ultimately, of polyketides, that are constructed by generating modifications in the megalomicin PKS so that the protein complexes produced have altered activities in one or more respects and thus produce polyketides other than the natural product of the PKS. Novel polyketides may thus be prepared, or polyketides in general prepared more readily, using this method. By providing a large number of

different genes or gene clusters derived from a naturally occurring PKS gene cluster, each of which has been modified in a different way from the native cluster, an effectively combinatorial library of polyketides can be produced as a result of the multiple variations in these activities. As will be further described below, the metes and bounds of this embodiment of the invention can be described on the polyketide, protein, and the encoding nucleotide sequence levels.

As described above, a modular PKS "derived from" the megalomicin or other naturally occurring PKS includes a modular PKS (or its corresponding encoding gene(s)) that retains the scaffolding of the utilized portion of the naturally occurring gene. Not all modules need be included in the constructs; however, the constructs can also comprise more than six modules. On the constant scaffold, at least one enzymatic activity is mutated, deleted, replaced, or inserted so as to alter the activity of the resulting PKS relative to the original (native) PKS. Alteration results when these activities are deleted or are replaced by a different version of the activity, or simply mutated in such a way that a polyketide other than the natural product results from these collective activities. This occurs because there has been a resulting alteration of the starter unit and/or extender unit, stereochemistry, chain length or cyclization, and/or reductive or dehydration cycle outcome at a corresponding position in the product polyketide. Where a deleted activity is replaced, the origin of the replacement activity may come from a corresponding activity in a different naturally occurring PKS or from a different region of the megalomicin PKS. Any or all of the megalomicin PKS genes may be included in the derivative or portions of any of these may be included, but the scaffolding of a functional PKS protein is retained in whatever derivative is constructed. The derivative preferably contains a thioesterase activity from the megalomicin or another PKS.

Thus, a PKS derived from the megalomicin PKS includes a PKS that contains the scaffolding of all or a portion of the megalomicin PKS. The derived PKS also contains at least two extender modules that are functional, preferably three extender modules, and more preferably four or more extender modules, and most preferably six extender modules. The derived PKS also contains mutations, deletions, insertions, or replacements of one or more of the activities of the functional modules of the megalomicin PKS so that the nature of the resulting

polyketide is altered at both the protein and DNA sequence levels. Particular preferred embodiments include those wherein a KS, AT, or ACP domain has been deleted or replaced by a version of the activity from a different PKS or from another location within the same PKS. Also preferred are derivatives where at least one non-condensation cycle enzymatic activity (KR, DH, or ER) has been deleted or added or wherein any of these activities has been mutated so as to change the structure of the polyketide synthesized by the PKS.

Conversely, also included within the definition of a PKS derived from the megalomicin PKS are functional non-megalomicin PKS modules or their encoding genes wherein at least one domain or coding sequence therefor of a megalomicin PKS module has been inserted. Exemplary is the use of the megalomicin AT for extender module 2, which accepts a methylmalonyl CoA extender unit rather than malonyl CoA, to replace a malonyl specific AT in another PKS. Other examples include insertion of portions of non-condensation cycle enzymatic activities or other regions of megalomicin synthase activity into a heterologous PKS at both the DNA and protein levels.

Thus, there are at least five degrees of freedom for constructing a hybrid PKS in terms of the polyketide that will be produced. First, the polyketide chain length is determined by the number of extender modules in the PKS, and the present invention includes hybrid PKSs that contain 6, as wells as fewer or more than 6, extender modules. Second, the nature of the carbon skeleton of the PKS is determined by the specificities of the acyl transferases that determine the nature of the extender units at each position, e.g., malonyl, methylmalonyl, ethylmalonyl, or other substituted malonyl. Third, the loading module specificity also has an effect on the resulting carbon skeleton of the polyketide. The loading module may use a different starter unit, such as acetyl, butyryl, and the like. As noted above, another method for varying loading module specificity involves inactivating the KS activity in extender module 1 (KS1) and providing alternative substrates, called diketides, that are chemically synthesized analogs of extender module 1 diketide products, for extender module 2. This approach was illustrated in PCT publication Nos. 97/02358 and 99/03986, incorporated herein by reference, wherein the KS1 activity was inactivated through mutation. Fourth, the oxidation state at various positions of the polyketide will be determined by the dehydratase and reductase

portions of the modules. This will determine the presence and location of ketone and alcohol moieties and C-C double bonds or C-C single bonds in the polyketide.

Finally, the stereochemistry of the resulting polyketide is a function of three aspects of the synthase. The first aspect is related to the AT/KS specificity associated with substituted malonyls as extender units, which affects stereochemistry only when the reductive cycle is missing or when it contains only a ketoreductase, as the dehydratase would abolish chirality. Second, the specificity of the ketoreductase may determine the chirality of any beta-OH. Finally, the enoylreductase specificity for substituted malonyls as extender units may influence the stereochemistry when there is a complete KR/DH/ER available.

Thus, the modular PKS systems generally and the megalomicin PKS system particularly permit a wide range of polyketides to be synthesized. As compared to the aromatic PKS systems, the modular PKS systems accept a wider range of starter units, including aliphatic monomers (acetyl, propionyl, butyryl, isovaleryl, and the like.), aromatics (aminohydroxybenzoyl), alicyclics (cyclohexanoyl), and heterocyclics (thiazolyl). Certain modular PKSs have relaxed specificity for their starter units (Kao *et al.*, 1994, *Science*, *supra*). Modular PKSs also exhibit considerable variety with regard to the choice of extender units in each condensation cycle. The degree of beta-ketoreduction following a condensation reaction can be altered by genetic manipulation (Donadio *et al.*, 1991, *Science*, *supra*; Donadio *et al.*, 1993, *Proc. Natl. Acad. Sci. USA* *90*: 7119-7123). Likewise, the size of the polyketide product can be varied by designing mutants with the appropriate number of modules (Kao *et al.*, 1994, *J. Am. Chem. Soc.* *116*:11612-11613). Lastly, modular PKS enzymes are particularly well known for generating an impressive range of asymmetric centers in their products in a highly controlled manner. The polyketides, antibiotics, and other compounds produced by the methods of the invention are typically single stereoisomeric forms. Although the compounds of the invention can occur as mixtures of stereoisomers, it may be beneficial in some instances to generate individual stereoisomers. Thus, the combinatorial potential within modular PKS pathways based on any naturally occurring modular, such as the megalomicin, PKS scaffold is virtually unlimited.

While hybrid PKSs are most often produced by "mixing and matching" portions of PKS coding sequences, mutations in DNA encoding a PKS can also be used to introduce, alter, or delete an activity in the encoded polypeptide. Mutations can be made to the native sequences using conventional techniques. The substrates for mutation can be an entire cluster of genes or only one or two of them; the substrate for mutation may also be portions of one or more of these genes. Techniques for mutation include preparing synthetic oligonucleotides including the mutations and inserting the mutated sequence into the gene encoding a PKS subunit using restriction endonuclease digestion. See, *e.g.*, Kunkel, 1985, *Proc. Natl. Acad. Sci. USA 82*: 448; Geisselsoder *et al.*, 1987, *BioTechniques 5*:786. Alternatively, the mutations can be effected using a mismatched primer (generally 10-20 nucleotides in length) that hybridizes to the native nucleotide sequence, at a temperature below the melting temperature of the mismatched duplex. The primer can be made specific by keeping primer length and base composition within relatively narrow limits and by keeping the mutant base centrally located. See Zoller and Smith, 1983, *Methods Enzymol. 100*:468. Primer extension is effected using DNA polymerase, the product cloned, and clones containing the mutated DNA, derived by segregation of the primer extended strand, selected. Identification can be accomplished using the mutant primer as a hybridization probe. The technique is also applicable for generating multiple point mutations. *See, e.g.*, Dalbie-McFarland *et al.*, 1982, *Proc. Natl. Acad. Sci. USA 79*: 6409. PCR mutagenesis can also be used to effect the desired mutations.

Random mutagenesis of selected portions of the nucleotide sequences encoding enzymatic activities can also be accomplished by several different techniques known in the art, e.g., by inserting an oligonucleotide linker randomly into a plasmid, by irradiation with X-rays or ultraviolet light, by incorporating incorrect nucleotides during *in vitro* DNA synthesis, by error-prone PCR mutagenesis, by preparing synthetic mutants, or by damaging plasmid DNA *in vitro* with chemicals, in accordance with the methods of the present invention. Chemical mutagens include, for example, sodium bisulfite, nitrous acid, nitrosoguanidine, hydroxylamine, agents which damage or remove bases thereby preventing normal base-pairing such as hydrazine or formic acid, analogues of nucleotide precursors such as 5-bromouracil, 2-aminopurine, or acridine

intercalating agents such as proflavine, acriflavine, quinacrine, and the like. Generally, plasmid DNA or DNA fragments are treated with chemical mutagens, transformed into *E. coli* and propagated as a pool or library of mutant plasmids.

In constructing a hybrid PKS of the invention, regions encoding enzymatic activity, i.e., regions encoding corresponding activities from different PKS synthases or from different locations in the same PKS, can be recovered, for example, using PCR techniques with appropriate primers. By "corresponding" activity encoding regions is meant those regions encoding the same general type of activity. For example, a KR activity encoded at one location of a gene cluster "corresponds" to a KR encoding activity in another location in the gene cluster or in a different gene cluster. Similarly, a complete reductase cycle could be considered corresponding. For example, KR/DH/ER can correspond to a KR alone.

If replacement of a particular target region in a host PKS is to be made, this replacement can be conducted *in vitro* using suitable restriction enzymes. The replacement can also be effected *in vivo* using recombinant techniques involving homologous sequences framing the replacement gene in a donor plasmid and a receptor region in a recipient plasmid. Such systems, advantageously involving plasmids of differing temperature sensitivities are described, for example, in PCT publication No. WO 96/40968, incorporated herein by reference. The vectors used to perform the various operations to replace the enzymatic activity in the host PKS genes or to support mutations in these regions of the host PKS genes can be chosen to contain control sequences operably linked to the resulting coding sequences in a manner such that expression of the coding sequences can be effected in an appropriate host.

However, simple cloning vectors may be used as well. If the cloning vectors employed to obtain PKS genes encoding derived PKS lack control sequences for expression operably linked to the encoding nucleotide sequences, the nucleotide sequences are inserted into appropriate expression vectors. This need not be done individually, but a pool of isolated encoding nucleotide sequences can be inserted into expression vectors, the resulting vectors transformed or transfected into host cells, and the resulting cells plated out into individual colonies. The invention provides a variety of recombinant DNA

compounds in which the various coding sequences for the domains and modules of the megalomicin PKS are flanked by non-naturally occurring restriction enzyme recognition sites.

The various PKS nucleotide sequences can be cloned into one or more recombinant vectors as individual cassettes, with separate control elements, or under the control of, e.g., a single promoter. The PKS subunit encoding regions can include flanking restriction sites to allow for the easy deletion and insertion of other PKS subunit encoding sequences so that hybrid PKSs can be generated. The design of such unique restriction sites is known to those of skill in the art and can be accomplished using the techniques described above, such as site-directed mutagenesis and PCR.

The expression vectors containing nucleotide sequences encoding a variety of PKS enzymes for the production of different polyketides are then transformed into the appropriate host cells to construct the library. In one straightforward approach, a mixture of such vectors is transformed into the selected host cells and the resulting cells plated into individual colonies and selected to identify successful transformants. Each individual colony has the ability to produce a particular PKS synthase and ultimately a particular polyketide. Typically, there will be duplications in some, most, or all of the colonies; the subset of the transformed colonies that contains a different PKS in each member colony can be considered the library. Alternatively, the expression vectors can be used individually to transform hosts, which transformed hosts are then assembled into a library. A variety of strategies are available to obtain a multiplicity of colonies each containing a PKS gene cluster derived from the naturally occurring host gene cluster so that each colony in the library produces a different PKS and ultimately a different polyketide. The number of different polyketides that are produced by the library is typically at least four, more typically at least ten, and preferably at least 20, and more preferably at least 50, reflecting similar numbers of different altered PKS gene clusters and PKS gene products. The number of members in the library is arbitrarily chosen; however, the degrees of freedom outlined above with respect to the variation of starter, extender units, stereochemistry, oxidation state, and chain length enables the production of quite large libraries.

Methods for introducing the recombinant vectors of the invention into suitable hosts are known to those of skill in the art and typically include the use of $CaCl_2$ or agents such as other divalent cations, lipofection, DMSO, protoplast transformation, conjugation, infection, transfection, and electroporation. The polyketide producing colonies can be identified and isolated using known techniques and the produced polyketides further characterized. The polyketides produced by these colonies can be used collectively in a panel to represent a library or may be assessed individually for activity.

The libraries of the invention can thus be considered at four levels: (1) a multiplicity of colonies each with a different PKS encoding sequence; (2) the proteins produced from the coding sequences; (3) the polyketides produced from the proteins assembled into a functional PKS; and (4) antibiotics or compounds with other desired activities derived from the polyketides. Of course, combination libraries can also be constructed wherein members of a library derived, for example, from the megalomicin PKS can be considered as a part of the same library as those derived from, for example, the rapamycin PKS or DEBS.

Colonies in the library are induced to produce the relevant synthases and thus to produce the relevant polyketides to obtain a library of polyketides. The polyketides secreted into the media can be screened for binding to desired targets, such as receptors, signaling proteins, and the like. The supernatants *per se* can be used for screening, or partial or complete purification of the polyketides can first be effected. Typically, such screening methods involve detecting the binding of each member of the library to receptor or other target ligand. Binding can be detected either directly or through a competition assay. Means to screen such libraries for binding are well known in the art and can be applied in accordance with the methods of the present invention. Alternatively, individual polyketide members of the library can be tested against a desired target. In this event, screens wherein the biological response of the target is measured can more readily be included. Antibiotic activity can be verified using typical screening assays such as those set forth in Lehrer *et al.*, 1991, *J. Immunol. Meth. 137*:167-173, incorporated herein by reference, and in the Examples below.

The invention provides methods for the preparation of a large number of polyketides. These polyketides are useful intermediates in formation of

compounds with antibiotic or other activity through hydroxylation, epoxidation, and glycosylation reactions as described above. In general, the polyketide products of the PKS must be further modified, typically by hydroxylation and glycosylation, to exhibit potent antibiotic activity. Hydroxylation results in the novel polyketides of the invention that contain hydroxyl groups at C-6, which can be accomplished using the hydroxylase encoded by the *eryF* gene, and/or C-12, which can be accomplished using the hydroxylase encoded by the *picK* or *eryK* gene. Also, the *oleP* gene is available in recombinant form, which can be used to express the *oleP* gene product in any host cell. A host cell, such as a *Streptomyces* host cell or a *Saccharopolyspora erythraea* host cell, modified to express the *oleP* gene thus can be used to produce polyketides comprising the C-8-C-8a epoxide present in oleandomycin. Thus the invention provides such modified polyketides. The presence of hydroxyl groups at these positions can enhance the antibiotic activity of the resulting compound relative to its unhydroxylated counterpart.

Methods for glycosylating polyketides are generally known in the art and can be applied in accordance with the methods of the present invention; the glycosylation may be effected intracellularly by providing the appropriate glycosylation enzymes or may be effected *in vitro* using chemical synthetic means as described herein and in PCT publication No. WO 98/49315, incorporated herein by reference. Preferably, glycosylation with desosamine, mycarose, and/or megosamine is effected in accordance with the methods of the invention in recombinant host cells provided by the invention. In general, the approaches to effecting glycosylation mirror those described above with respect to hydroxylation. The purified enzymes, isolated from native sources or recombinantly produced may be used *in vitro*. Alternatively and as noted, glycosylation may be effected intracellularly using endogenous or recombinantly produced intracellular glycosylases. In addition, synthetic chemical methods may be employed.

The antibiotic modular polyketides may contain any of a number of different sugars, although D-desosamine, or a close analog thereof, is most common. Erythromycin, picromycin, megalomicin, narbomycin, and methymycin contain desosamine. Erythromycin also contains L-cladinose (3-O-methyl mycarose). Tylosin contains mycaminose (4-hydroxy desosamine), mycarose and

6-deoxy-D-allose. 2-acetyl-1-bromodesosamine has been used as a donor to glycosylate polyketides by Masamune *et al.*, 1975, *J. Am. Chem. Soc. 97*: 3512-3513. Other, apparently more stable donors include glycosyl fluorides, thioglycosides, and trichloroacetimidates; see Woodward *et al.*, 1981, *J. Am. Chem. Soc.* 103: 3215; Martin *et al.*, 1997, *J. Am. Chem. Soc. 119*: 3193; Toshima *et al.*, 1995, *J. Am. Chem. Soc. 117*: 3717; Matsumoto *et al.*, 1988, *Tetrahedron Lett. 29*: 3575. Glycosylation can also be effected using the polyketide aglycones as starting materials and using *Saccharopolyspora erythraea* or *Streptomyces venezuelae* or other host cell to make the conversion, preferably using mutants unable to synthesize macrolides, as discussed in the preceding Section.

Thus, a wide variety of polyketides can be produced by the hybrid PKS enzymes of the invention. These polyketides are useful as antibiotics and as intermediates in the synthesis of other useful compounds, as described in the following section.

Section VII: Host Cells Containing Multiple Expression Vectors

A recombinant host cell of the invention may contain nucleic acid encoding a megalomicin PKS domain, module, or protein, or megalomicin modification enzyme at a single genetic locus, *e.g.*, on a single plasmid or at a single chromosomal locus, or at different genetic loci, *e.g.*, on separate plasmids and/or chromosomal loci. By "multiple" is meant two or more; by "vector" is meant a nucleic acid molecule which can be used to transform host systems and which contains an independent expression system containing a coding sequence under control of a promoter and optionally a selectable marker and any other suitable sequences regulating expression. Typical such vectors are plasmids, but other vectors such as phagemids, cosmids, viral vectors and the like can be used according to the nature of the host. Of course, one or more of the separate vectors may integrate into the chromosome of the host (selection may not be required for maintenance of integrated vectors).

In one embodiment, the invention provides a recombinant host cell, which comprises at least two separate autonomously replicating recombinant DNA expression vectors, each of said vectors comprises a recombinant DNA compound encoding a megalomicin PKS domain or a megalomicin modification enzyme

operably linked to a promoter. In another embodiment, the invention provides a recombinant host cell, which comprises at least one autonomously replicating recombinant DNA expression vector and at least one modified chromosome, each of said vector(s) and each of said modified chromosome comprises a recombinant DNA compound encoding a megalomicin PKS domain or a megalomicin modification enzyme operably linked to a promoter. Preferably, the autonomously replicating recombinant DNA expression vector and/or the modified chromosome further comprises distinct selectable markers.

The above multiple-vector (chromosome) expression systems can also be used for expressing heterogeneous polyketide biosynthetic enzymes, *e.g.*, for expressing *Micromonospora megalomicea* megalomicin PKS protein, module, or domain or a megalomicin modification enzyme with a PKS protein, module, or domain, or modification enzyme from other origins in the same host cells. By placing various activities on different expression vectors, a high degree of variation can be achieved in an efficient manner. A variety of hosts can be used; any suitable host cell that can maintain multiple vectors can readily be used. Preferred hosts include *Streptomyces*, yeast, *E. coli,* other actinomycetes, and plant cells, and mammalian or insect cells or other suitable recombinant hosts can also be used. Preferred among yeast strains are *Saccharomyces cerevisiae* and *Pichia pastoris*. Preferred actinomycetes include various strains of *Streptomyces*.

If one chooses to use a host cell that does not naturally produce a polyketide, then one may need to ensure that the recombinant host is modified to also contain a holo ACP synthase activity that effects pantetheinylation of the acyl carrier protein. See PCT Pub. No. WO 97/13845, incorporated herein by reference. One of the multiple vectors may be used for this purpose. This activation step is necessary for activation of the ACP. The expression system for the holo ACP synthase may be supplied on a vector separate from that carrying a PKS coding sequence or may be supplied on the same vector or may be integrated into the chromosome of the host, or may be supplied as an expression system for a fusion protein with all or a portion of a polyketide synthase (see U.S. Patent No. 6,033,883, incorporated herein by reference).

It should be noted that in some recombinant hosts, it may also be necessary to activate the polyketides produced through postsynthesis modifications when

polyketides having such modifications are desired. If this is the case for a particular host, the host will be modified, for example by transformation, to contain those enzymes necessary for effecting these modifications. Among such enzymes, for example, are glycosylation enzymes. The use of multiple vectors can facilitate the introduction of expression systems for such enzymes.

In a preferred embodiment, the multiple vector system is used to assemble rapidly and efficiently a combinatorial library of polyketides and the PKS/modification enzymes that produce them. In an illustrative embodiment, the multiple vector system comprises four different vectors, one comprising the *megAI* gene, one the *megAII* gene, one the *megAIII* gene, and one the modification enzyme(s) gene(s). Each of these vectors can be modified to make a set of vectors. For example, one set could contain all possible AT substitutions in the loading and first and second extender modules of the megAI gene product. Another set could contain expression systems for a variety of different modification enzymes. With these four vectors sets and by combining each member of each set with each member of the other three sets, a very large library of cells, vector sets, and polyketides can be rapidly and efficiently assembled.

The combinatorial potential of a modular PKS such as the megalomicin PKS (ignoring the additional potential of different modification enzyme systems) is minimally given by: AT_L X $(AT_E \text{ X } 4)_M$ where AT_L is the number of loading acyl transferases, AT_E is the number of extender acyl transferases, and M is the number of modules in the gene cluster. The number 4 is present in the formula because this represents the number of ways a keto group can be modified by either 1) no reaction; 2) KR activity alone; 3) KR+DH activity; or 4) KR+DH+ER activity. It has been shown that expression of only the first two modules of the erythromycin PKS resulted in the production of a predicted truncated triketide product (See Kao et al., *J. Am. Chem. Soc.*, 116:11612-11613 ((1994)). A novel 12-membered macrolide similar to methymycin aglycone was produced by expression of modules 1-5 of this PKS in *S. coelicolor* (See Kao et al., *J. Am. Chem. Soc.*, 117:9105-9106 (1995)). This work shows that PKS modules are functionally independent so that lactone ring size can be controlled by the number of modules present.

In addition to controlling the number of modules, the modules can be genetically modified, for example, by the deletion of a ketoreductase domain as described by Donadio et al., *Science*, 252:675-679 (1991); and Donadio et al., *Gene*, 115:97-103 (1992). In addition, the mutation of an enoyl reductase domain was reported by Donadio, et al., *Proc. Natl. Acad. Sci.*, 90:7119-7123 (1993). These modifications also resulted in modified PKS and thus modified polyketides.

As stated above, in the present invention, the coding sequences for catalytic activities derived from the megalomicin PKS systems found in nature can be used in their native forms or modified by standard mutagenesis techniques to delete or diminish activity or to introduce an activity into a module in which it was not originally present. For example, a KR activity can be introduced into a module normally lacking that function.

In one embodiment of the invention herein, a single host cell is modified to contain a multiplicity of vectors, each vector contributing a portion of the synthesis of a megalomicin PKS and modification enzyme (if any) system. Each of the multiple vectors for production of the megalomicin PKS system typically encodes at least two modules, and at least one of the vectors integrates into the chromosome of the host. Integration can be effected using suitable phage or integrating vectors or by homologous recombination. If homologous recombination is used, the integration event may also be designed to delete endogenous PKS genes residing in the chromosome, as described in the PCT application WO 95/08548. In these embodiments, too, a selectable marker such as hygromycin or thiostrepton resistance can be included in the vector that effects integration.

As mentioned above, additional enzymes that effect post-translational modifications to the enzyme systems in the megalomicin PKS may be introduced into the host through suitable recombinant expression systems. In addition, enzymes that activate the polyketides themselves, for example, through glycosylation may be added. It may also be desirable to modify the cell to produce more of a particular substrate utilized in polyketide biosynthesis. For example, it is generally believed that malonyl CoA levels in yeast are higher than methylmalonyl CoA; if yeast is chosen as a host, it may be desirable to increase

methylmalonyl CoA levels by the addition of one or more biosynthetic enzymes therefor.

The multiple-vector expression system can also be used to make polyketides produced by the addition of synthetic starter units to a PKS that contains an inactivated ketosynthase (KS) in the first module. As noted above, this modification permits the system to incorporate a suitable diketide thioester such as 3-hydroxy-2-methyl pantonoic acid-N-acetyl cysteamine thioester, or similar thioesters of diketide analogs, as described by Jacobsen et al., *Science*, 277:367-369 (1997). The construction of PKS modules containing inactivated ketosynthase regions can be conducted by methods known in the art, such as the method described in U.S. Patent No. 6,080,555 and PCT publication Nos. WO 99/03986 and 97/02358, each of which is incorporated herein by reference, in accordance with the methods of the present invention.

The multiple-vector expression system can be used to produce polyketides in hosts that normally do not produce them, such as *E. coli* and yeast. It also provides more efficient means to provide a variety of polyketide products by supplying the elements of the introduced PKS, whether in an *E. coli* or yeast host or in other more traditionally used hosts, such as *Streptomyces*. The invention also includes libraries of polyketides prepared using the methods of the invention.

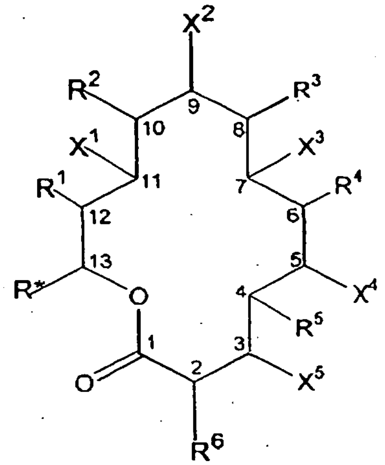
Section VIII: Compounds

The methods and recombinant DNA compounds of the invention are useful in the production of polyketides. In one important aspect, the invention provides methods for making antibiotic compounds related in structure to erythromycin, a potent antibiotic compound. The invention also provides novel ketolide compounds, polyketide compounds with potent antibiotic activity of significant interest due to activity against antibiotic resistant strains of bacteria. See Griesgraber *et al.*, 1996, *J. Antibiot. 49*: 465-477, incorporated herein by reference. Most if not all of the ketolides prepared to date are synthesized using erythromycin A, a derivative of 6-dEB, as an intermediate. In one embodiment, the present invention provides the 3-keto derivatives of the megalomicins for use as antibiotics. In particular, the 3-keto derivative of megalomicin A is a preferred ketolide of the invention. These compounds can be made chemically, substantially

in accordance with the procedures for making ketolides described in the prior art, or in recombinant host cells of the invention in which the megosamine and desosamine biosynthetic and transferase genes are present but which do not make or transfer the mycarose moiety and/or the PKS has been modified to delete the KR domain of extender module 6. The invention also provides methods for making intermediates useful in preparing traditional, 6-dEB- and erythromycin-derived ketolide compounds. See Griesgraber *et al., supra*; Agouridas *et al.*, 1998, *J. Med. Chem. 41*: 4080-4100, U.S. Patent Nos. 5,770,579; 5,760,233; 5,750,510; 5,747,467; 5,747,466; 5,656,607; 5,635,485; 5,614,614; 5,556,118; 5,543,400; 5,527,780; 5,444,051; 5,439,890; 5,439,889; and PCT publication Nos. WO 98/09978 and 98/28316, each of which is incorporated herein by reference.

As noted above, the hybrid PKS genes of the invention can be expressed in a host cell that contains the desosamine, megosamine, and/or mycarose biosynthetic genes and corresponding transferase genes as well as the required hydroxylase gene(s), which may, for example and without limitation, be either *picK*, *megK*, or *eryK* (for the C-12 position) and/or *megF* or*eryF* (for the C-6 position). The resulting compounds have antibiotic activity but can be further modified, as described in the patent publications referenced above, to yield a desired compound with improved or otherwise desired properties. Alternatively, the aglycone compounds can be produced in the recombinant host cell, and the desired glycosylation and hydroxylation steps carried out *in vitro* or *in vivo*, in the latter case by supplying the converting cell with the aglycone, as described above.

The compounds of the invention are thus optionally glycosylated forms of the polyketide set forth in formula (1) below which are hydroxylated at either the C-6 or the C-12 or both. The compounds of formula (1) can be prepared using the loading and the six extender modules of a modular PKS, modified or prepared in hybrid form as herein described. These polyketides have the formula:



(2)

including the glycosylated and isolated stereoisomeric forms thereof;

wherein R* is a straight chain, branched or cyclic, saturated or unsaturated substituted or unsubstituted hydrocarbyl of 1-15C;

each of R^1-R^6 is independently H or alkyl (1-4C) wherein any alkyl at R^1 may optionally be substituted;

each of X^1-X^5 is independently two H, H and OH, or =O; or

each of X^1-X^5 is independently H and the compound of formula (2) contains a double-bond in the ring adjacent to the position of said X at 2-3, 4-5, 6-7, 8-9 and/or 10-11;

with the proviso that:

at least two of R^1-R^6 are alkyl (1-4C).

Preferred compounds comprising formula 2 are those wherein at least three of R^1-R^5 are alkyl (1-4C), preferably methyl or ethyl; more preferably wherein at least four of R^1-R^5 are alkyl (1-4C), preferably methyl or ethyl. Also preferred are those wherein X^2 is two H, =O, or H and OH, and/or X^3 is H, and/or X^1 is OH and/or X^4 is OH and/or X^5 is OH. Also preferred are compounds with variable R* when R^1-R^5 is methyl, X^2 is =O, and X^1, X^4 and X^5 are OH. The glycosylated forms (i.e., mycarose or cladinose at C-3, desosamine at C-5, and/or megosamine at C-6) of the foregoing are also preferred.

As described above, there are a wide variety of diverse organisms that can modify compounds such as those described herein to provide compounds with or that can be readily modified to have useful activities. For example, *Saccharopolyspora erythraea* can convert 6-dEB to a variety of useful

compounds. The compounds provided by the present invention can be provided to cultures of *Saccharopolyspora erythraea* and converted to the corresponding derivatives of erythromycins A, B, C, and D in accordance with the procedure provided in the Examples, below. To ensure that only the desired compound is produced, one can use an *S. erythraea eryA* mutant that is unable to produce 6-dEB but can still carry out the desired conversions (Weber *et al.*, 1985, *J. Bacteriol.* *164*(1): 425-433). Also, one can employ other mutant strains, such as *eryB*, *eryC*, *eryG*, and/or *eryK* mutants, or mutant strains having mutations in multiple genes, to accumulate a preferred compound. The conversion can also be carried out in large fermentors for commercial production. Each of the erythromycins A, B, C, and D has antibiotic activity, although erythromycin A has the highest antibiotic activity. Moreover, each of these compounds can form, under treatment with mild acid, a C-6 to C-9 hemiketal with motilide activity. For formation of hemiketals with motilide activity, erythromycins B, C, and D, are preferred, as the presence of a C-12 hydroxyl allows the formation of an inactive compound that has a hemiketal formed between C-9 and C-12.

Thus, the present invention provides the compounds produced by hydroxylation and glycosylation of the compounds of the invention by action of the enzymes endogenous to *Saccharopolyspora erythraea* and mutant strains of *S. erythraea*. Such compounds are useful as antibiotics or as motilides directly or after chemical modification. For use as antibiotics, the compounds of the invention can be used directly without further chemical modification. Erythromycins A, B, C, and D all have antibiotic activity, and the corresponding compounds of the invention that result from the compounds being modified by *Saccharopolyspora erythraea* also have antibiotic activity. These compounds can be chemically modified, however, to provide other compounds of the invention with potent antibiotic activity. For example, alkylation of erythromycin at the C-6 hydroxyl can be used to produce potent antibiotics (clarithromycin is C-6-O-methyl), and other useful modifications are described in, for example, Griesgraber *et al.*, 1996, *J. Antibiot.* *49*: 465-477, Agouridas *et al.*, 1998, *J. Med. Chem.* *41*: 4080-4100, U.S. Patent Nos. 5,770,579; 5,760,233; 5,750,510; 5,747,467; 5,747,466; 5,656,607; 5,635,485; 5,614,614; 5,556,118; 5,543,400; 5,527,780;

5,444,051; 5,439,890; and 5,439,889; and PCT publication Nos. WO 98/09978 and 98/28316, each of which is incorporated herein by reference.

For use as motilides, the compounds of the invention can be used directly without further chemical modification. Erythromycin and certain erythromycin analogs are potent agonists of the motilin receptor that can be used clinically as prokinetic agents to induce phase III of migrating motor complexes, to increase esophageal peristalsis and LES pressure in patients with GERD, to accelerate gastric emptying in patients with gastric paresis, and to stimulate gall bladder contractions in patients after gallstone removal and in diabetics with autonomic neuropathy. See Peeters, 1999, Motilide Web Site, http://www.med.kuleuven.ac.be/med/gih/motilid.htm, and Omura *et al.*, 1987, Macrolides with gastrointestinal motor stimulating activity, *J. Med. Chem. 30*: 1941-3). The corresponding compounds of the invention that result from the compounds of the invention being modified by *Saccharopolyspora erythraea* also have motilide activity, particularly after conversion, which can also occur *in vivo*, to the C-6 to C-9 hemiketal by treatment with mild acid. Compounds lacking the C-12 hydroxyl are especially preferred for use as motilin agonists. These compounds can also be further chemically modified, however, to provide other compounds of the invention with potent motilide activity.

Moreover, and also as noted above, there are other useful organisms that can be employed to hydroxylate and/or glycosylate the compounds of the invention. As described above, the organisms can be mutants unable to produce the polyketide normally produced in that organism, the fermentation can be carried out on plates or in large fermentors, and the compounds produced can be chemically altered after fermentation. In addition to *Saccharopolyspora erythraea*, *Streptomyces venezuelae*, *S. narbonensis*, *S. antibioticus*, *Micromonospora megalomicea*, *S. fradiae*, and *S. thermotolerans* can also be used. In addition to antibiotic activity, compounds of the invention produced by treatment with *M. megalomicea* enzymes can have antiparasitic activity as well. Thus, the present invention provides the compounds produced by hydroxylation and glycosylation by action of the enzymes endogenous to *S. erythraea*, *S. venezuelae*, *S. narbonensis*, *S. antibioticus*, *M. megalomicea*, *S. fradiae*, and *S. thermotolerans*.

The present invention also provides methods and genetic constructs for producing the glycosylated and/or hydroxylated compounds of the invention directly in the host cell of interest. Thus, the recombinant genes of the invention, which include recombinant *megAI*, *megAII*, and *megAIII* genes with one or more deletions and/or insertions, including replacements of a *megA* gene fragment with a gene fragment from a heterologous PKS gene, can be included on expression vectors suitable for expression of the encoded gene products in *Saccharopolyspora erythraea*, *Micromonospora megalomicea*, *S. venezuelae*, *S. narbonensis*, *S. antibioticus*, *S. fradiae*, and *S. thermotolerans*.

The compounds of the invention can be produced by growing and fermenting the host cells of the invention under conditions known in the art for the production of other polyketides. The compounds of the invention can be isolated from the fermentation broths of these cultured cells and purified by standard procedures. The compounds can be readily formulated to provide the pharmaceutical compositions of the invention. The pharmaceutical compositions of the invention can be used in the form of a pharmaceutical preparation, for example, in solid, semisolid, or liquid form. This preparation will contain one or more of the compounds of the invention as an active ingredient in admixture with an organic or inorganic carrier or excipient suitable for external, enteral, or parenteral application. The active ingredient may be compounded, for example, with the usual non-toxic, pharmaceutically acceptable carriers for tablets, pellets, capsules, suppositories, solutions, emulsions, suspensions, and any other form suitable for use.

The carriers which can be used include water, glucose, lactose, gum acacia, gelatin, mannitol, starch paste, magnesium trisilicate, talc, corn starch, keratin, colloidal silica, potato starch, urea, and other carriers suitable for use in manufacturing preparations, in solid, semi-solid, or liquified form. In addition, auxiliary stabilizing, thickening, and coloring agents and perfumes may be used. For example, the compounds of the invention may be utilized with hydroxypropyl methylcellulose essentially as described in U.S. Patent No. 4,916,138, incorporated herein by reference, or with a surfactant essentially as described in EPO patent publication No. 428,169, incorporated herein by reference.

Oral dosage forms may be prepared essentially as described by Hondo *et al.*, 1987, *Transplantation Proceedings XIX*, Supp. 6: 17-22, incorporated herein by reference. Dosage forms for external application may be prepared essentially as described in EPO patent publication No. 423,714, incorporated herein by reference. The active compound is included in the pharmaceutical composition in an amount sufficient to produce the desired effect upon the disease process or condition.

For the treatment of conditions and diseases caused by infection, a compound of the invention may be administered orally, topically, parenterally, by inhalation spray, or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvant, and vehicles. The term parenteral, as used herein, includes subcutaneous injections, and intravenous, intramuscular, and intrasternal injection or infusion techniques.

Dosage levels of the compounds of the invention are of the order from about 0.01 mg to about 50 mg per kilogram of body weight per day, preferably from about 0.1 mg to about 10 mg per kilogram of body weight per day. The dosage levels are useful in the treatment of the above-indicated conditions (from about 0.7 mg to about 3.5 mg per patient per day, assuming a 70 kg patient). In addition, the compounds of the invention may be administered on an intermittent basis, i.e., at semi-weekly, weekly, semi-monthly, or monthly intervals.

The amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated and the particular mode of administration. For example, a formulation intended for oral administration to humans may contain from 0.5 mg to 5 gm of active agent compounded with an appropriate and convenient amount of carrier material, which may vary from about 5 percent to about 95 percent of the total composition. Dosage unit forms will generally contain from about 0.5 mg to about 500 mg of active ingredient. For external administration, the compounds of the invention may be formulated within the range of, for example, 0.00001% to 60% by weight, preferably from 0.001% to 10% by weight, and most preferably from about 0.005% to 0.8% by weight.

It will be understood, however, that the specific dose level for any particular patient will depend on a variety of factors. These factors include the

activity of the specific compound employed; the age, body weight, general health, sex, and diet of the subject; the time and route of administration and the rate of excretion of the drug; whether a drug combination is employed in the treatment; and the severity of the particular disease or condition for which therapy is sought.

A detailed description of the invention having been provided above, the following examples are given for the purpose of illustrating the invention and shall not be construed as being a limitation on the scope of the invention or claims.

Example 1

Cloning and Characterization of the Megalomicin Biosynthetic Gene Cluster from *Micromonospora meglomicea*

Experimental Procedures

Bacterial Strains, Media, and Growth Conditions

Routine DNA manipulations were performed in *Escherichia coli* XL1 Blue or *E. coli* XL1 Blue MR (Stratagene) using standard culture conditions (Sambrook *et al.*, 1989). *M. megalomicea* subs. *nigra* NRRL3275 was obtained from the ATCC collection and cultured according to recommended protocols. For isolation of genomic DNA, *M. megalomicea* was grown in TSB (Hopwood *et al.*, 1985) at 30 °C. *S. lividans* K4-114 (Ziermann and Betlach, 1999), which carries a deletion of the actinorhodin biosynthetic gene cluster, was used as the host for expression of the *megAI-AIII* genes. *S. lividans* strains were maintained on R5 agar at 30°C and grown in liquid YEME for preparation of protoplasts (Hopwood *et al.*, 1985). *S. erythraea* NRRL2338 was used for expression of the megosamine genes. *S. erythraea* strains were maintained on R5 agar at 34°C and grown in liquid TSB for preparation of protoplasts.

Manipulation of DNA and Organisms

Manipulation and transformation of DNA in *E. coli* was performed by standard procedures (Sambrook *et al.*, 1989) or by suppliers protocols. Protoplasts of *S. lividans* and *S. erythraea* were generated for transformation by plasmid DNA using the standard procedure. *S. lividans* transformants were selected on R5 using 2 ml of a 0.5 mg/ml thiostrepton overlay. *S. erythraea* transformants were selected on R5 using 1.5 ml of a 0.6 mg/ml apramycin overlay.

Isolation of the meg gene cluster

A cosmid library was prepared in SuperCos (Stratagene) from *M. megalomicea* total DNA partially digested with *Sau*3A I, and introduced into *E. coli* using a Gigapack III XL (Stratagene) *in-vitro* packaging kit. ^{32}P-labelled DNA probes encompassing the KS2 domain from *ery* DEBS, or a mixture of segments encompassing modules 1 and 2 from *ery* DEBS were used separately to screen the cosmid library by colony hybridization. Several colonies which hybridized with the probes were further analyzed by sequencing the ends of their cosmid inserts using T3 and T7 primers. BLAST (Altschul *et al.*, 1990) analysis of the sequences revealed several colonies with DNA sequences highly homologous to genes from the ery cluster. Together with restriction analysis, this led to the isolation of two overlapping cosmids, pKOS079-93A and pKOS079-93D which covered ~45 kb of the *meg* cluster. A 400 bp PCR fragment was generated from the left end of and pKOS079-93D and used to reprobe the cosmid library. Likewise, a 200 bp PCR fragment generated from the right end of pKOS079-93A was used to reprobe the cosmid library. Analysis of hybridizing colonies as described above resulted in identification of two additional cosmids, pKOS079-138B and pKOS79-124B which overlap the previous two cosmids. BLAST analysis of the far left and right end sequences of these cosmids indicated no homology to any known genes related to polyketide biosynthesis and therefore indicates that the set of four cosmids spans the entire megalomicin biosynthetic gene cluster.

DNA sequencing and analysis

PCR-based double stranded DNA sequencing was performed on a Beckman CEQ 2000 capillary sequencer using reagents and protocols provided by the manufacturer. A shotgun library of the entire cosmid pKOS079-93D insert was made as follows: DNA was first digested with *Dra* I to eliminate the vector fragment, then partially digested with *Sau*3A I. After agarose electrophoresis, bands between 1-3 kb were excised from the gel and ligated with *Bam*H I digested pUC19. Another shotgun library was generated from a 12 kb *Xho* I/*Eco*R I fragment subcloned from cosmid pKOS079-93A to extend the sequence to the *megF* gene. A 4 kb *Bgl* II/ *Xho* I fragment from cosmid pKOS079-138B was

sequenced by primer walking to extend the sequencing to the *megT* gene. Sequence was assembled using Sequencher (Gene Codes Corp.) software package and analyzed with MacVector (Oxford Molecular Group) and the NCBI BLAST server (www.ncbi.nlm.nih.gov/BLAST/).

Plasmids

Plasmid pKOS108-6 is a modified version of pKAO127'kan' (Ziermann and Betlach, 1999; Ziermann and Betlach, 2000) in which the *ery*AI-III genes between the *Pac* I and *Eco*R I sites have been replaced with the *meg*AI-III genes. This was done by first substituting a synthetic nucleotide DNA duplex (5'-TAAGAATTCGGAGATCTGGCCTCAGCTCTAGAC (SEQ ID NO: 21), complementary oligo 5'-AATTGTCTAGAGCTGAGGCCAGATCTCCGAATTCTTAAT (SEQ ID NO: 22)) between the *Pac* I and *Eco*R I sites of the pKAO127'kan' vector fragment. The 22 kb *Eco*R I/*Bgl* II fragment from cosmid pKOS079-93D containing the *megAI-II* genes was inserted into *Eco*R I and *Bgl* II sites of the resulting plasmid to generate pKOS024-84. A 12 kb *Bgl* II/*Bbv*C I fragment containing the *megAIII* and part of the *megCII* gene was subcloned from pKOS079-93A and excised as a *Bgl* II/*Xba* I fragment and ligated into the corresponding sites of pKOS024-84 to yield the final expression plasmid pKOS108-06.

The megosamine integrating vector, pKOS97-42, was constructed as follows: A subclone was generated containing the 4 kb *Xho* I/*Sca* I fragment from pKOS79-138B together with the 1.7 kb *Sca* I/*Pst* I fragment from pKOS79-93D in Litmus 28 (Stratagene). The entire 5.7 kb fragment was then excised as a *Spe* I/*Pst* I fragment and combined with the 6.3 kb *Pst* I/*Eco*R I fragment from KOS79-93D and *Eco*R I/*Xba* I digested pSET152 (Bierman *et al.*, 1992) to construct plasmid pKOS97-42.

Production and analysis of secondary metabolites

Fermentation for production of polyketide, LC/MS analysis, and quantification of 6-dEB for *S. lividans* K4-114/pKOS108-6 and *S. lividans* K4-114/pKAO127'kan' were essentially as previously described (Xue *et al.*, 1999). *S. erythraea* NRRL2338 and *S. erythraea*/pKOS97-42 were grown for 6 days in F1

media (Brünker *et al.*, 1998). Samples of broth were clarified in a microcentrifuge (5 min, 13,000 rpm). For LC/MS preparation, isopropanol was added to the supernatant (1:2 ratio) and centrifuged again. Erythromycins and megalomicins were detected by electrospray mass spectrometry and quantity was determined by evaporative light scattering detection (ELSD). The LC retention time and mass spectra of erythromycin and megalomicins were identical to known standards.

Nucleotide sequence of the meg *gene cluster*

A series of 4 overlapping inserts containing the *meg* cluster (Figure 9) were isolated from a cosmid library prepared from total genomic DNA of *M. megalomicea* and covers > 100 kb of the genome. A contiguous 48 kb segment which encodes the megalomicin PKS and several deoxysugar biosynthetic genes was sequenced and analyzed. The segment contains 17 complete ORFs as well as an incomplete ORF at each end, organized as shown in Figure 9.

PKS genes. The ORFs *megAI*, *megAII* and *megAIII* encode the polyketide synthase responsible for synthesis of 6-dEB. The enzyme complex, *meg* DEBS, is highly similar to *ery* DEBS, with each of the three predicted polypeptides sharing an average of 83% overall similarity with their *ery* PKS counterpart. Both PKSs are composed of 6 modules (2 modules per polypeptide) and each module is organized in the identical manner (Figure 9). A dendrogram analysis (Schwecke *et al.*, 1995) employing 70 acyltranferase (AT) domains revealed that the 6 *meg* extender AT domains cluster with AT domains that incorporate methylmalonyl CoA (not shown). The loading module of *meg* DEBS also lacks a KS^Q domain which is utilized by most macrolide PKSs for decarboxylation of the starter unit to initiate polyketide synthesis (Bisang *et al.*, 1999; Kuhstoss *et al.*, 1996; Kakavas *et al.*, 1997; Xue *et al.*, 1998), implying that priming begins with a propionate unit. In addition, a conserved Gly to Pro substitution in the NADPH-binding region of the ketoreductase (KR) domain of module 3 is observed in *meg* DEBS, which has been proposed to account for its inactivity in *ery* DEBS (Donadio *et al.*, 1991).

Deoxysugar genes. BLAST (Altschul *et al.*, 1990) analysis of the genes flanking the PKS indicated that 12 complete ORFs and 1 partial ORF appear to encode functions required for synthesis of one of the three megalomicin deoxysugars. Assignment of each ORF to a specific deoxysugar pathway was

made based on comparison to the *ery* genes and other related genes involved in deoxysugar biosynthesis (Table 2).

Table 2. Deduced functions of genes identified in the megalomicin gene cluster.

Gene	*Closest Match (polypeptide)*[a]	*% Sim*[a]	*Proposed Pathway*	*Proposed Function*	*Reference*
megT	EryBVI		Mycarose/ Megosamine	2,3-Dehydratase	(Summers *et al.*, 1997; Gaisser *et al.*, 1997)
megDVI	EryCII	63	Megosamine	3,4-Isomerase	(Summers *et al.*, 1997)
megDI	EryCIII	79	Megosamine	Glycosyltransferase	(Summers *et al.*, 1997)
megY	AcyA (*S. thermotolerans*)	52		Mycarose *O*-acyl-transferase	(Arisawa *et al.*, 1994)
megDII	EryCI	58	Megosamine	Aminotransferase	(Dhillon *et al.*, 1989; Summers *et al.*, 1997)
megDIII	DesVI (*S. venezuelae*)	61	Megosamine	Dimethyltransferase	(Xue *et al.*, 1998)
megDIV	DmnU (*S. peucetius*)	65	Megosamine	3,5-Epimerase	(Olano *et al.*, 1999)
megDV	Dehydrogenase (*A. orientalis*)	61	Megosamine	4-Ketoreductase	(Summers *et al.*, 1997; van Wageningen *et al.*, 1998)
megDVII	EryBII	73	Megosamine	2,3-Reductase	(Summers *et al.*, 1997)
megBV	EryBV	86	Mycarose	Glycosyltransferase	(Summers *et al.*, 1997; Gaisser *et al.*, 1997)
megBIV	EryBIV	80	Mycarose	4-Ketoreductase	(Summers *et al.*, 1997; Gaisser *et al.*, 1997)
megAI	EryAI	81	6-dEB	Polyketide Synthase	(Donadio and Katz, 1992)
megAII	EryAII	85	6-dEB	Polyketide Synthase	(Donadio and Katz, 1992)
megAIII	EryAIII	83	6-dEB	Polyketide Synthase	(Donadio and Katz, 1992)
megCII	EryCII	82	Desosamine	3,4-Isomerase	(Summers *et al.*, 1997)
meg CIII	EryCIII	89	Desosamine	Glycosylyltransferase	(Summers *et al.*, 1997)
megBII	EryBII	87	Mycarose	2,3-Reductase	(Summers *et al.*, 1997)
megH	EryH	84		Thioesterase	(Haydock *et al.*, 1991)
megF	EryF			C-6 Hydroxylase	(Weber *et al.*, 1991)

a. Determined by BLASTX analysis using default parameters.

Three ORFs, *megBV*, *megCIII* and *megDI*, encode glycosyltransferases, apparently one for attachment of each deoxysugar to the macrolide. MegBV was most similar to EryBV, the erythromycin mycarosyltransferase, and hence was assigned to the mycarose pathway in the *meg* cluster. The closest match for both of the remaining glycosyltransferases was EryCIII, the desosaminyltransferase in erythromycin biosynthesis. Given the higher degree of similarity between EryCIII and MegCIII (Table 2), MegCIII was designated the desosaminyltransferase, leaving MegDI as the proposed megosaminyltransferase. In similar fashion, assignments were made accordingly for; MegCII and MegDVI, two putative 3,4-isomerases similar to EryCII; MegBII and MegDVII, 2,3-reductases homologous to EryBII; MegBIV and MegDV, putative 4-ketoreductases similar to EryBIV (Table 2). The remaining ORFs involved in deoxysugar biosynthesis, *megT*, *megDII*, *megDIII* and *megDIV*, each encode a putative 2,3-dehydratase, aminotransferase, dimethyltransferase and 3,5-epimerase, respectively (Table 2). Since both the megosamine and desosamine pathways require an aminotransferase and a dimethyltransferase, and since mycarose and megosamine each require a 2,3-dehydratase and a 3,5-epimerase, assignments of these four genes to a specific pathway could not be made on the basis of sequence comparison alone. However, the latter three are implicated in megosamine biosynthesis by experiments described below.

Other genes. Two additional complete ORFs, designated *megY* and *megH* and an incomplete ORF, designated *megF*, were also identified in the cluster. MegH and MegF share high degrees of similarity with EryH and EryF. EryH and homologs in other macrolide gene clusters are thioesterase-like proteins with unknown function in polyketide gene clusters (Haydock *et al.*, 1991; Xue *et al.*, 1998; Butler *et al.*, 1999; Tang *et al.*, 1999). EryF encodes the erythronolide B C-6 hydroxylase (Figure 8) (Weber *et al.*, 1991; Andersen and Hutchinson, 1992). MegY does not have an *ery* counterpart but appears to belong to a (small) family of *O*-acyltransferases that transfer short acyl chains to macrolides. Two classes exist: AcyA and MdmB transfer acetyl or propionyl groups to the C-3 hydroxyls on 16-membered macrolide rings (Arisawa *et al.*, 1994; Hara and Hutchinson, 1992); CarE and Mpt transfer isovalerate or propionate to the mycarosyl moiety of carbomycin and midecamycin, respectively (Epp *et al.*, 1989; Arisawa *et al.*, 1993;

Gu *et al.*, 1996). The structures of various megalomicins suggest that MegY belongs to the latter class and is the acyltransferase which converts megalomicin A to megalomicins B, C1, or C2 (verified experimentally below).

Heterologous expression of the meg *PKS genes.*

The wild type and genetically modified versions of the *ery* DEBS have been used extensively in heterologous *Streptomyces* hosts for enzyme studies and the production of novel polyketide compounds. Given the similarities between the *ery* and *meg* DEBSs, production characteristics were compared in a commonly used *Streptomyces* host strain. The three *meg*A ORFs were cloned into the expression plasmid pKAO127'kan' (Ziermann and Betlach, 1999) in place of the *ery*A ORFs. Both plasmids, pKAO127'kan' encoding *ery* DEBS and pKOS108-06 encoding *meg* DEBS, were introduced in *Streptomyces lividans* K4-114 and the production of 6-dEB was determined in shake-flask fermentations. The production profiles were similar in both cases and the maximum titer of 6-dEB was between 30-40 mg/L. In addition, both PKSs produced small amounts (~5%) of 8,8a-deoxyoleandolide, which results from the priming of the PKS with acetate instead of propionate (Kao *et al.*, 1994b). This observation indicates that the loading AT domains of the PKSs display similar relaxed specificities towards starter units.

Conversion of erythromycin to megalomicin in S. erythraea.

An examination of the *meg* cluster revealed that the putative megosamine biosynthetic genes are clustered directly upstream of the PKS genes. If the hypothesis that these genes are sufficient for biosynthesis and attachment of megosamine to an erythromycin intermediate is correct, then functional expression of these genes in a strain which produces erythromycin, such as *S. erythraea*, should result in production of megalomicin. A 12 kb DNA fragment carrying all the genes between the leftmost *Xho*I site and the *Eco*RI site (Figure 9) was integrated in the chromosome of *S. erythraea* using the site-specific integrating vector pSET152 (Bierman *et al.*, 1992). It was surmised that the left and right ends of this fragment would contain necessary promoter regions for transcription of the convergent set of genes in *M. megalomicea* and that they would likely operate in *S. erythraea*.

Fermentation broth from *S. erythraea*/KOS97-42, which contains the integrated *meg* genes, was analyzed by LC/MS and compared to LC/MS profiles of the parent *S. erythraea* strain without the *meg* genes, as well as to megalomicin standards purified from *M. megalomicea*. The new strain was found to produce a mixture of erythromycin A and various megalomicins (~4:1 ratio), thereby showing that the predicted megosamine biosynthetic and glycosyltransferase genes are contained within the cloned *meg* fragment. The two most abundant congeners identified were megalomicins B and C1. Megalomicin A and C2 were also detected in smaller amounts. The presence of the megalomicins B, C1 and C2 also provides direct evidence for the function of the *O*-acyl transferase, MegY, which is present in the integrated *meg* fragment.

Discussion

The homologies observed among modular PKSs enabled the use of *ery* PKS genes to clone the *meg* biosynthetic gene cluster from *M. megalomicea*. The close similarities between the megalomicin and erythromycin biosynthetic pathways is also reflected in the overall organization of their genes and in the high degree of homology of the corresponding individual gene-encoded polypeptides. Production of 6-dEB from *meg* DEBS in *S. lividans* and conversion of erythromycin to megalomicin using the *megD* genes in *S. erythraea* provides direct evidence that the identified gene cluster is responsible for synthesis of megalomicin.

As seen in Figure 9, the ~ 40 kb segments of the two clusters beginning with *ery/megBV* on the left through the *ery/megF* genes retain a nearly identical organizational arrangement. The notable differences in this region are *eryG* and IS*1136* which are absent from the segment of the *meg* cluster analyzed. The *eryG* gene encodes an S-adenosylmethionine (SAM)-dependent mycarosyl methyltransferase that converts erythromycin C to erythromycin A (Figure 8) (Weber *et al.*, 1990; Haydock *et al.*, 1991). The mycarose moiety is modified by esterification (MegY) in megalomicin biosynthesis (Figure 8) and, therefore, the absence of an *eryG* homolog would be expected in the *meg* cluster. The IS*1136* element located between *eryAI* and *eryAII* (Donadio and Staver, 1993) is not

known to play a role in erythromycin biosynthesis and its origin in the *ery* cluster has not been determined.

Upstream of the common *meg/eryBIV* and *BV* genes, the gene clusters diverge. The ~ 6 kb segment between *eryBV* and *eryK*, the left border of the *ery* gene cluster (Pereda *et al.*, 1997), contains the remaining genes required for mycarose (*eryBVI* and *BVII*) and desosamine biosynthesis (*eryCIV, CV*, and *CVI*) and the C-12 hydroxylase (*eryK*) (Stassi *et al.*, 1993). In contrast, the region upstream of *megBV* encodes a set of genes (*megDI-DVII* and *megY*) which can account for all the activities unique to megalomicin biosynthesis (Figure 9). Since introduction of this *meg* DNA segment into *S. erythraea* results in production of megalomicins, it is clear that these genes encode the functions for TDP-megosamine biosynthesis and transfer to its putative substrate erythromycin C, and to acylate megalomicin A (Figure 8). The remaining region upstream of *megDVI* should therefore encode genes only for mycarose and desosamine biosynthesis.

Olano *et al.* (Olano *et al.*, 1999) have recently described a pathway for biosynthesis of TDP-L-daunosamine, a deoxysugar component of the antitumor compounds daunorubicin and doxorubicin produced by *Streptomyces peucetius*. Their pathway proposes four steps from the intermediate TDP-4-keto-6-deoxyglucose controlled by the gene cluster *dnmJQTUVZ*, although the functions for *dnmQ* and *dnmZ* could not be identified and the precise order of reactions in the pathway could not be determined. The genes *dnmT*, *dnmU*, *dnmJ* and *dnmV* each have proposed counterparts in the *meg* cluster, *megT*, *megDIV*, *megDII*, and *megDV*, respectively (see Figure 10)

It is possible to describe a pathway to convert TDP-2,6-dideoxy-3,4-diketo-D-hexose (or its enol tautomer), the last intermediate common to the mycarose and megosamine pathways, to TDP-megosamine through the sequence of 5-epimerization, 4-ketoreduction, 3-amination, and 3-*N*-dimethylation employing the genes *megDIV*, *megDV, megDII,* and *megDIII*. This employs the same functions proposed for biosynthesis of TDP-daunosamine by Olano *et al.*, but in a different sequential order. However, it does not account for the *megDVI* and *megDVII* genes since their activities are not required for this route. A parallel pathway which employs these genes is also shown in Figure 10. In this alternate route, 2,3-reduction and 3,4-tautomerization are performed by the *megDVII* and

megDVI gene products, respectively. A unified single pathway that employs both 4-ketoreduction (*megDV*) and 2,3-reduction (*megDVII*) could not be determined. Because the entire gene set from *megDVI* through *megDVII* was introduced in *S. erythraea* to produce TDP-megosamine, it is not possible to determine which, if either, of the two alternative pathways is operative, but this can be addressed through systematic gene disruption and complementation.

The 48 kb segment sequenced also contains genes required for synthesis of TDP-L-mycarose and TDP-D-desosamine (Fig 10). For the latter, *megCII*, which encodes a putative 3,4-isomerase, the first step in the committed TDP-desosamine pathway, appears to be translationally coupled to *megAIII*, almost exactly as its erythromycin counterpart, *eryCII*, was found translationally coupled to *eryAIII* (Summers *et al.*, 1997). The high degree of similarity between MegCII and EryCII suggests that the pathway to desosamine in the megalomicin- and erythromycin-producing organisms are most likely the same. Similarly, the finding that *megBII* and *megBIV*, encoding a 2,3-reductase and 4-ketoreductase, contain close homologs in the mycarose pathway for erythromycin also suggests that TDP-L-mycarose synthesis in the two host organisms is the same.

Of interest are the two genes that encode putative 2,3-reductases, *megBII* and *megDVII*. Because MegBII most closely resembles EryBII, a known mycarose biosynthetic enzyme (Weber *et al.*, 1990), and because *megBII* resides in the same location of the *meg* cluster as its counterpart in the *ery* cluster, *megBII* is assigned to the mycarose pathway and *megDVII* to the megosamine pathway. Furthermore, the lower degree of similarity between MegDVII and either EryBII or MegBII (Table 2) provides a basis for assigning the opposite L and D isomeric substrates to each of the enzymes (Figure 10). Finally, *megT*, which encodes a putative 2,3-dehydratase, is also related to a gene in the *ery* mycarose pathway, *eryBVI*. In *S. erythraea*, the proposed intermediate generated by EryBVI represents the first committed step in the biosynthesis of mycarose (Figure 10). However, the proposed pathways in Figure 10 suggest this may be an intermediate common to both mycarose and megosamine biosynthesis in *M. megalomicea*. Therefore, *megT* is named following the designation of the equivalent gene in the daunosamine pathway, *dnmT* (Olano *et al.*, 1999)

The preferred host-vector system for expression of *meg* DEBS described here has been used previously for the heterologous expression of modular PKS genes from the erythromycin (Kao *et al.*, 1994a; Ziermann and Betlach, 1999), picromycin (Tang *et al.*, 1999) and oleandomycin pathways, as well as for the generation of novel polyketide backbones where domains have been removed, added or exchanged in various combinations (McDaniel *et al.*, 1999). Recently, hybrid polyketides have been generated through the co-expression of subunits from different PKS systems (Tang *et al.*, 2000).

Expression of the *megDVI-megDVII* segment in *S. erythraea* and the corresponding production of megalomicins in this host establishes the likely order of sugar attachment in megalomicin synthesis. Furthermore, it provides a means to produce megalomicin in a more genetically friendly host organism, leading to the creation of megalomicin analogs by manipulating the PKS. Over 60 6-dEB analogs have been produced by combinatorial biosynthesis using the *ery* PKS (McDaniel *et al.*, 1999; Xue *et al.*, 1999). The titers of megalomicin could also be significantly increased above the 5 mg/L obtained from *M. megalomiciea* by introducing the genes into an industrially optimized strain of *S. erythraea*, many of which can produce as much as 10 g/L of erythromycin.

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Example 2

Stabilizing meg PKS Expression Plasmid by Codon Engineering

Materials and methods

All bacterial strains were cultured and transformed as described in Example 1.

Fermentation of Streptomyces and diketide feeding

Primary *Streptomyces* transformants were picked and placed in 6 mL of TSB liquid medium with 50 μg/L of thiostrepton and grown at 30°C. When the culture showed some growth (3-4days), it was transferred into a 250 mL flask containing 50 mL of R6 medium (pH 7.0) with 25 ug/L of thiostrepton and 1g/L of diketide ((2s,3R)2-methyl-3-hydroxyhexanoate N-propionyl cysteamine thioester) and placed in a 30°C incubator for 7 days.

Changing codons and making plasmids

There are several identical sequences in the coding sequences for module 2 and module 6 of the megalomicin PKS gene cluster. Expression plasmids containing the full length megalomicin PKS appeared to be somewhat unstable and subject to deletion in $recA^{+}$ strains like ET124567 and *Streptomyces* by intra-plasmid homologous recombination. To prevent significant homologous recombination and so stabilize expression plasmids, the codons of two regions of the module 6 coding sequence that are identical to regions in the module 2 coding sequence were changed without changing the sequence of protein encoded. The two regions changed in module 6 were from the 26739th base to 27,267th base and from position 27,697th base to 27,987th base, which were identical to the region from position 6810th base to 7338thbase and regions from position 7778thbase to 8068th base, respectively. The start codon of the loading domain of the meg PKS was set to be the 1st base. These sequences are shown below

```
>  6810-7338 Sequence in Module 2
TTGCAGCGGTTGTCGGTGGCGGTGCGGGAGGGGCGTCGGGTGTTGGGTGTGGTGGTGGGT
TCGGCGGTGAATCAGGATGGGGCGAGTAATGGGTTGGCGGCGCCGTCGGGGGTGGCGCAG
CAGCGGGTGATTCGGCGGGCGTGGGGTCGTGCGGGTGTGTCGGGTGGGGATGTGGGTGTG
GTGGAGGCGCATGGGACGGGGACGCGGTTGGGGGATCCGGTGGAGTTGGGGGCGTTGTTG
GGGACGTATGGGGTGGGTCGGGGTGGGGTGGGTCCGGTGGTGGTGGGTTCGGTGAAGGCG
AATGTGGGTCATGTGCAGGCGGCGGCGGGTGTGGTGGGTGTGATCAAGGTGGTGTTGGGG
TTGGGTCGGGGGTTGGTGGGTCCGATGGTGTGTCGGGGTGGGTTGTCGGGGTTGGTGGAT
TGGTCGTCGGGTGGGTTGGTGGTGGCGGATGGGGTGCGGGGGTGGCCGGTGGGTGTGGAT
GGGGTGCGTCGGGGTGGGGTGTCGGCGTTTGGGGTGTCGGGGACGAAT (SEQ ID NO: 23)
>  26736-27267 Sequence in Module 6
CTGCAGCGGTTGTCGGTGGCGGTGCGGGAGGGGCGTCGGGTGTTGGGTGTGGTGGTGGGT
TCGGCGGTGAATCAGGATGGGGCGAGTAATGGGTTGGCGGCGCCGTCGGGGGTGGCGCAG
CAGCGGGTGATTCGGCGGGCGTGGGGTCGTGCGGGTGTGTCGGGTGGGGATGTGGGTGTG
GTGGAGGCGCATGGGACGGGGACGCGGTTGGGGGATCCGGTGGAGTTGGGGGCGTTGTTG
GGGACGTATGGGGTGGGTCGGGGTGGGGTGGGTCCGGTGGTGGTGGGTTCGGTGAAGGCG
AATGTGGGTCATGTGCAGGCGGCGGCGGGTGTGGTGGGTGTGATCAAGGTGGTGTTGGGG
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TTGGGTCGGGGGTTGGTGGGTCCGATGGTGTGTCGGGGTGGGTTGTCGGGGTTGGTGGAT
TGGTCGTCGGGTGGGTTGGTGGTGGCGGATGGGGTGCGGGGGTGGCCGGTGGGTGTGGAT
GGGGTGCGTCGGGGTGGGGTGTCGGCGTTTGGGGTGTCGGGGACGAAT (SEQ ID NO: 24)
\> 26736-27267 Sequence with Codon Changes
CTGCAGCGCCTCTCCGTCGCCGTCCGCGAGGGCCGCCGAGTCCTCGGCGTCGTCGTCGGC
TCGGCCGTCAACCAAGACGGCGCGTCAAACGGCCTCGCCGCGCCCTCCGGCGTCGCCCAG
CAGCGCGTCATACGCCGCGCGTGGGACGCGCCGGAGTATCGGGCGGCGACGTCGGAGTC
GTCGAGGCCCACGGCACCGGCACCCGCCTCGGGGATCCCGTCGAGCTGGGCGCCCTCCTG
GGCACGTACGGCGTCGGCCGCGGCGGCGTCGGCCCGGTCGTCGTCGGCAGCGTCAAGGCC
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\> 6978-7337 Sequence in Module 2
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TGGTGCGCGGGTGGTGGCGTTGCGGGCGCGGGCGTTGCGGGCGTTGGCCGG (SEQ ID NO: 26)
\> 27697-27987 Sequence in Module 6
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\> 27697-27987 Sequence with Codon Changes
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CGGCGCCCGCGTCGTGGCCCTGCGCGCCCGCGCCCTGCGCGCCCTGGCCGG (SEQ ID NO: 28)

Three pieces of DNA from the two regions above were synthesized and verified by Retrogen, and the synthesized DNAs were cloned into pCR-Blunt II –TOPO, as shown in the Table 3 below.

Table 3. Plasmids containing synthesized DNA

Plasmids	Cloning sites and positions in meg PKS
pKOS97-1613	PstI-BamHI, 26,739th-26,947th base
PKOS97-1622	BamHI-BsmI, 26,947th –27,267th base
PKOS97-1628	SfaNI-FseI, 27,697th- 27,987th base

Assembly of the expression plasmid

First, ligation of the PstI-BamHI fragment of pKOS97-1613, the BamHI-BsmI fragment of pKOS97-1622 and BsmI-PstI linearized pKOS97-90 produced

pKOS97-151. Then, the insertion of the SfaNI-FseI fragment of pKOS97-1628 into pKOS97-151 gave rise to pKSO97-152. Then, the PstI-BlpI fragment of pKOS97-125 was used to replace the PstI-BlpI fragment of pKOS97-90a and produced pKOS97-160.

The final expression plasmid (in pRM5) pKOS97-162 was the result of BglII-NheI fragment of pKOS97-160 inserted into BglII-NheI sites of pKOS108-04.

Another expression plasmid pKOS97-152a was made by a four-fragment ligation. The four fragments were a BlpI-XbaI fragment (containing a cos site) of pKOS97-92a, a BglII-PstI fragment of pKOS97-81, a PstI-BlpI fragment of pKOS97-152, and a BglII-XbaI fragment of pKOS108-04 (as the vector).

Tests of the constructed plasmids showed that the plasmids containing the modified coding sequences were more stable than plasmids containing unmodified coding sequence.

Example 3

Construction of Ole-Meg Hybrid PKS

Construction of pRM1-based pKOS098-48 for the expression of OlePKS modules 1-4.

The 240-bp fragment containing the 3'-end portion of *oleAII* gene (at nt 11210-11452; the first base of the start codon of *oleAII* is nt 1) was PCR amplified with primers N98-38-1 (5'GAACAACTCCTGTCT<u>GCGGCCGC</u>G-3') (SEQ ID NO: 29) and N98-38-3 (5'-CG<u>GAATTC</u>**TCTAGAG**TCACGTCTCCAACCGCTTGTCGAGG-3') (SEQ ID NO: 30). The fragment contains a naturally occurring NotI site at its 5'-end and the engineered XbaI (bold) and EcoRI sites (underline) at its 3'-end following the *oleAII* stop codon. pKOS38-189 was digested with EcoRI and NotI to give five fragments of 8 kb, 5 kb, 4 kb, 2.5 kb and 2 kb. The 8-kb EcoRI-NotI fragment containing *oleAII* gene nt 2961 to nt 11210 and the 240-bp NotI, EcoRI treated PCR fragment were ligated into litmus 28 at the EcoRI site via a three-fragment ligation to give pKOS98-46. The 8.2-kb EoRI fragment from pKOS98-46 was cloned into pKOS38-174, a pRM1 derived plasmid containing *oleAI* and nt 1 to nt 2960 of *oleAII* to give pKOS98-48.

Construction of pSET152-based pKOS98-60 for the expression of megPKS modules 5-6.

The 360-bp fragment containing nt 1 to nt 366 of *megAIII* was PCR amplified with primers N98-40-3 (5'-TCTAGACTTAATTAA**GGAGGA**CA*CATATG*AGCGA-GAGCAGC-GGCATGACCG-3') (SEQ ID NO: 31) and N98-40-2 (5'- AACGCCTCCCAG-GAGATCTCCAGCA-3') (SEQ ID NO: 32). A PacI site and a NdeI site as well as the ribosome binding site were introduced at the 5'-end of the *megAI* start codon. The 360-bp PacI-BglII fragment was inserted into pKOS108-06 replacing the 22-kb PacI-BglII fragment to yield pKOS98-55. The 10-kb PacI-XbaI fragment containing megAIII gene and the annealed oligos N98-23-1 (5'-AATTCATAGCCTAGGT-3') (SEQ ID NO: 33) and N98-23-2 (5'-CTAGACCTAGGCTATG-3') (SEQ ID NO: 34) were ligated to PacI and EcoRI treated pSET152 derivative pKOS98-14 via a three-fragment ligation to give pKOS98-60.

Example 4

Conversion of Erythronolides to Erythromycins

A sample of a polyketide (~50 to 100 mg) is dissolved in 0.6 mL of ethanol and diluted to 3 mL with sterile water. This solution is used to overlay a three day old culture of *Saccharopolyspora erythraea* WHM34 (an *eryA* mutant) grown on a 100 mm R2YE agar plate at 30°C. After drying, the plate is incubated at 30°C for four days. The agar is chopped and then extracted three times with 100 mL portions of 1% triethylamine in ethyl acetate. The extracts are combined and evaporated. The crude product is purified by preparative HPLC (C-18 reversed phase, water-acetonitrile gradient containing 1% acetic acid). Fractions are analyzed by mass spectrometry, and those containing pure compound are pooled, neutralized with triethylamine, and evaporated to a syrup. The syrup is dissolved in water and extracted three times with equal volumes of ethyl acetate. The organic extracts are combined, washed once with saturated aqueous $NaHCO_3$, dried over Na_2SO_4, filtered, and evaporated to yield ~0.15 mg of product. The product is a glycosylated and hydroxylated compound corresponding to

erythromycin A, B, C, and D but differing therefrom as the compound provided differed from 6-dEB.

Example 5

Measurement of Antibacterial Activity

Antibacterial activity is determined using either disk diffusion assays with *Bacillus cereus* as the test organism or by measurement of minimum inhibitory concentrations (MIC) in liquid culture against sensitive and resistant strains of *Staphylococcus pneumoniae*.

Example 6

Evaluation of Antiparasitic Activity

Compounds can initially screened *in vitro* using cultures of *P. falciparum* FCR-3 and K1 strains, then *in vivo* using mice infected with *P. berghei*. Mammalian cell toxicity can be determined in FM3A or KB cells. Compounds can also be screened for activity against *P. berhei*. Compounds are also tested in animal studies and clinical trials to test the antiparasitic activity broadly (antimalarial, trypanosomiasis and Leishmaniasis).

The invention having now been described by way of written description and example, those of skill in the art will recognize that the invention can be practiced in a variety of embodiments and that the foregoing description and examples are for purposes of illustration and not limitation of the following claims.

Claims

1. An isolated nucleic acid comprising a nucleotide sequence encoding a domain of megalomicin polyketide synthase (PKS) or a megalomicin modification enzyme.

2. The isolated nucleic acid of claim 1, which encodes a PKS open reading frame (ORF) selected from the group consisting of megAI, megAII and megAIII.

3. The isolated nucleic acid of claim 1, wherein the PKS domain is selected from the group consisting of a TE domain, a KS domain, an AT domain, an ACP domain, a KR domain, a DH domain, and an ER domain.

4. The isolated nucleic acid of claim 1, wherein the nucleic acid comprises the coding sequence for a loading module, a thioesterase domain, and all six extender modules of megalomicin PKS.

5. The isolated nucleic acid of claim 1, which encodes a megalomicin modification enzyme that is involved in the conversion of 6-dEB into a megalomicin.

6. The isolated nucleic acid of claim 5, which encodes a megalomicin modification enzyme that is involved in the biosynthesis of mycarose, megosamine or desosamine.

7. The isolated nucleic acid of claim 1, wherein the nucleic acid codons of homologous regions within the PKS or the megalomicin modification enzyme coding sequence have been changed to reduce or abolish the homology without changing the amino acid sequences encoded by said changed nucleic acid codons.

8. The isolated nucleic acid of claim 1, which isolated nucleic acid fragment hybridizes to a nucleic acid having a nucleotide sequence set forth in the SEQ. ID NO:1.

9. A polypeptide, which is encoded by the isolated nucleic acid fragment of claim 1.

10. A recombinant DNA expression vector, comprising the isolated nucleic acid of claim 1 operably linked to a promoter.

11. A recombinant host cell, comprising the recombinant DNA expression vector of claim 10.

12. The recombinant host cell of claim 11, which is a *Streptomyces* or *Saccharopolyspora* host cell.

13. A recombinant host cell of claim 11, which comprises:

a) at least two separate autonomously replicating recombinant DNA expression vectors, each of said vectors comprises a recombinant DNA compound encoding a megalomicin PKS domain or a megalomicin modification enzyme operably linked to a promoter; or

b) at least one autonomously replicating recombinant DNA expression vector and at least one modified chromosome, each of said vector(s) and each of said modified chromosome comprises a recombinant DNA compound encoding a megalomicin PKS domain or a megalomicin modification enzyme operably linked to a promoter.

14. A hybrid PKS that comprises a polypeptide of claim 9 and is composed of at least a portion of a megalomicin PKS and at least a portion of a second PKS for a polyketide other than megalomicin.

15. The hybrid PKS of claim 14, wherein the second PKS is selected from the group consisting of a narbonolide PKS, an oleandolide PKS, and a DEBS PKS.

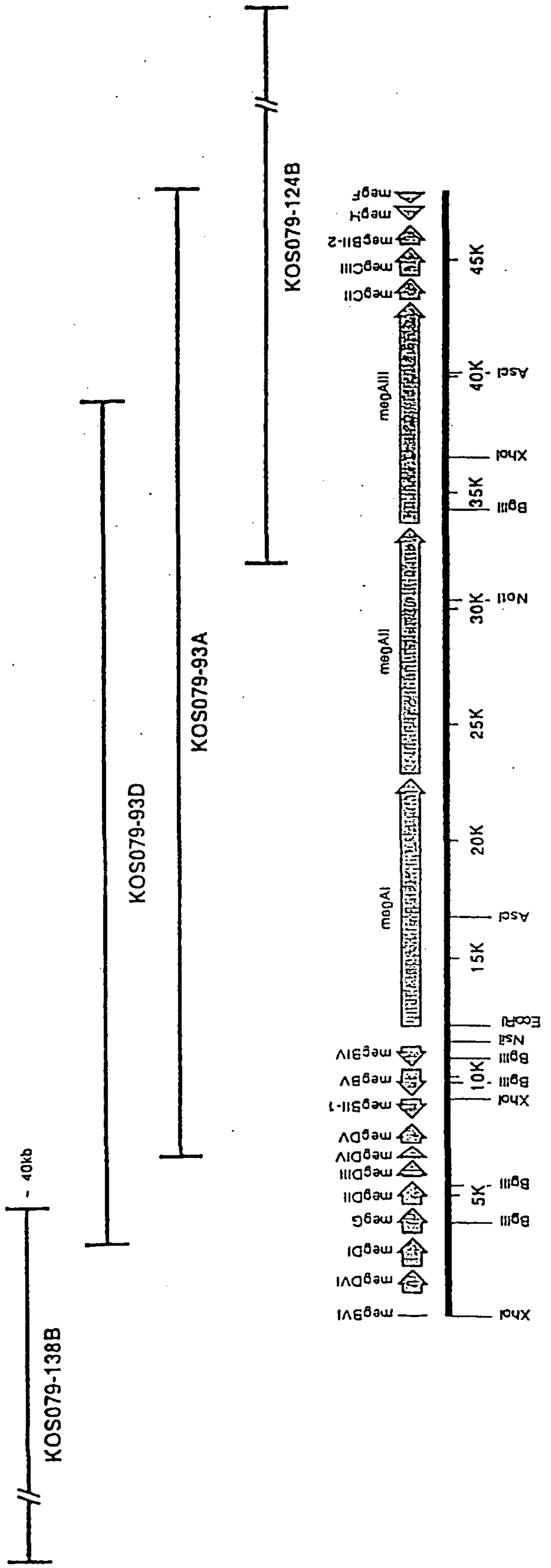
16. The hybrid PKS of claim 15 that is composed of the megAI and megAII gene products and the oleAIII gene product.

17. The hybrid PKS of claim 16, wherein the KS domain of module 1 of the megAI gene product has been inactivated by mutation.

18. A method of producing a polyketide, which method comprises growing the recombinant host cell of claim 11 under conditions whereby the megalomicin PKS domain encoded by the recombinant expression vector is produced and the polyketide is synthesized by the cell, and recovering the synthesized polyketide.

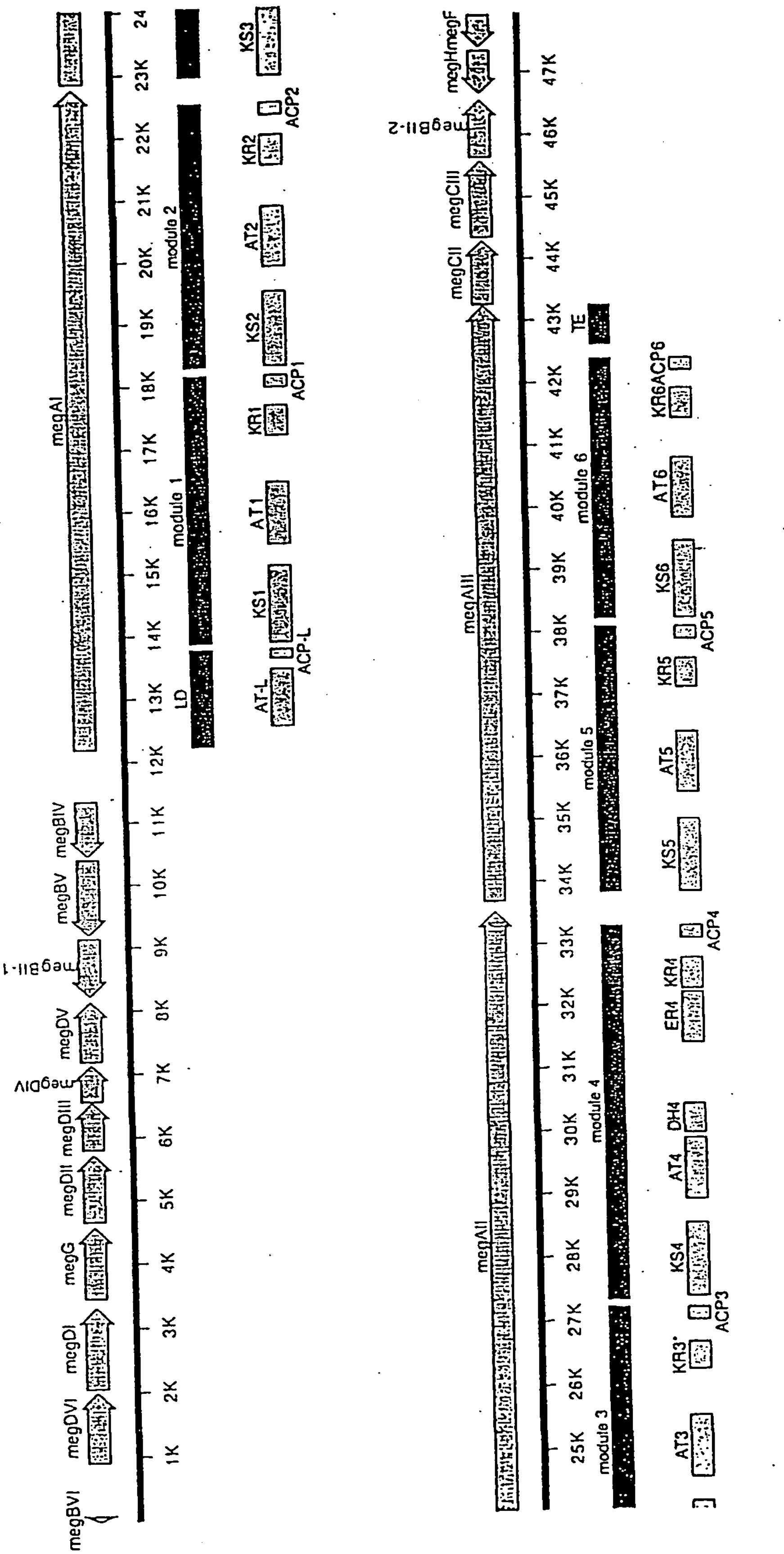
19. A recombinant host cell that comprises a recombinant expression vector that encodes a megalomicin modification enzyme.

20. The recombinant host cell of claim 19 that produces megosamine and can attach megosamine to a polyketide, wherein said host cell, in its naturally occurring non-recombinant state cannot produce megosamine.



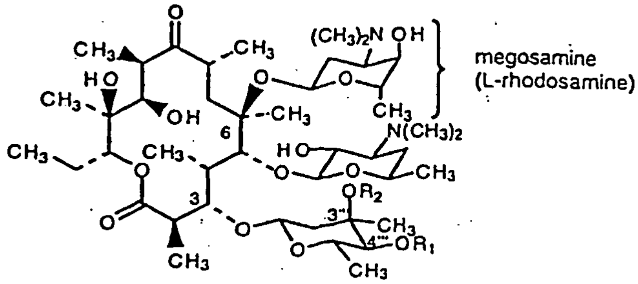
Cosmid Inserts

Figure 1

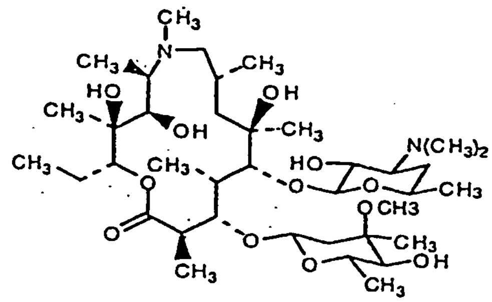


Megalomicin Biosynthetic Genes

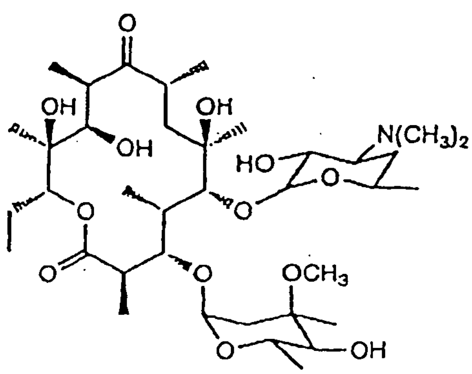
Figure 2



	R_1	R_2
Megalomicin A	H	H
B	$COCH_3$	H
C1	$COCH_3$	$COCH_3$
C2	$COCH_2CH_3$	$COCH_3$



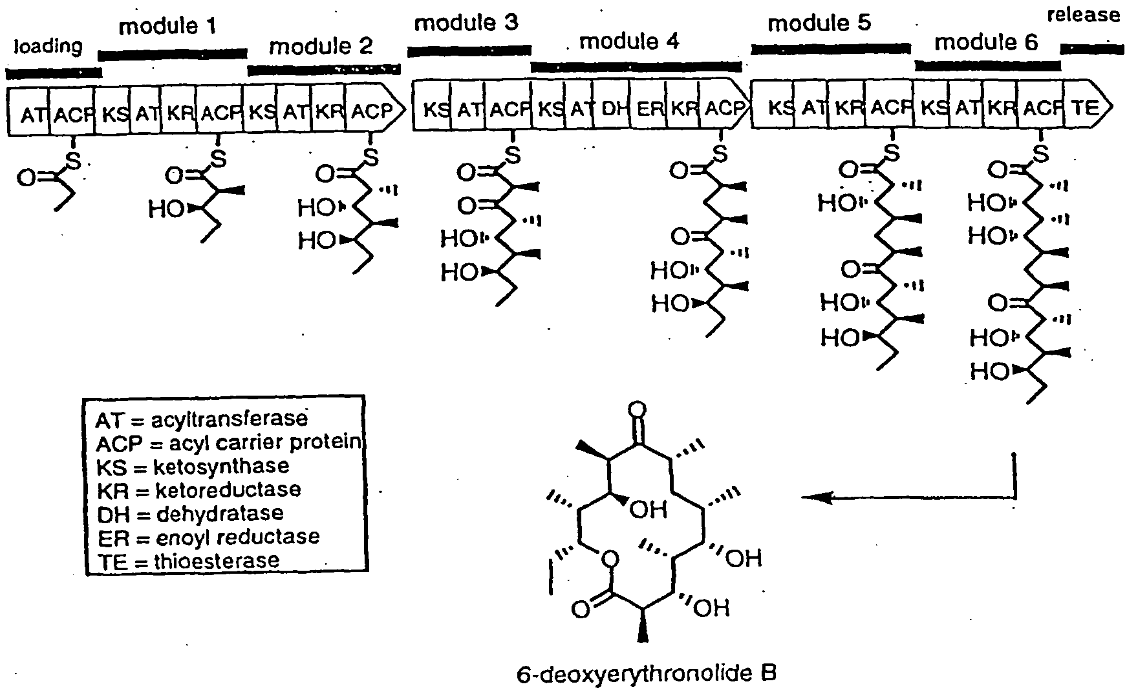
azithromycin
(Azithromax)



Erythromycin A

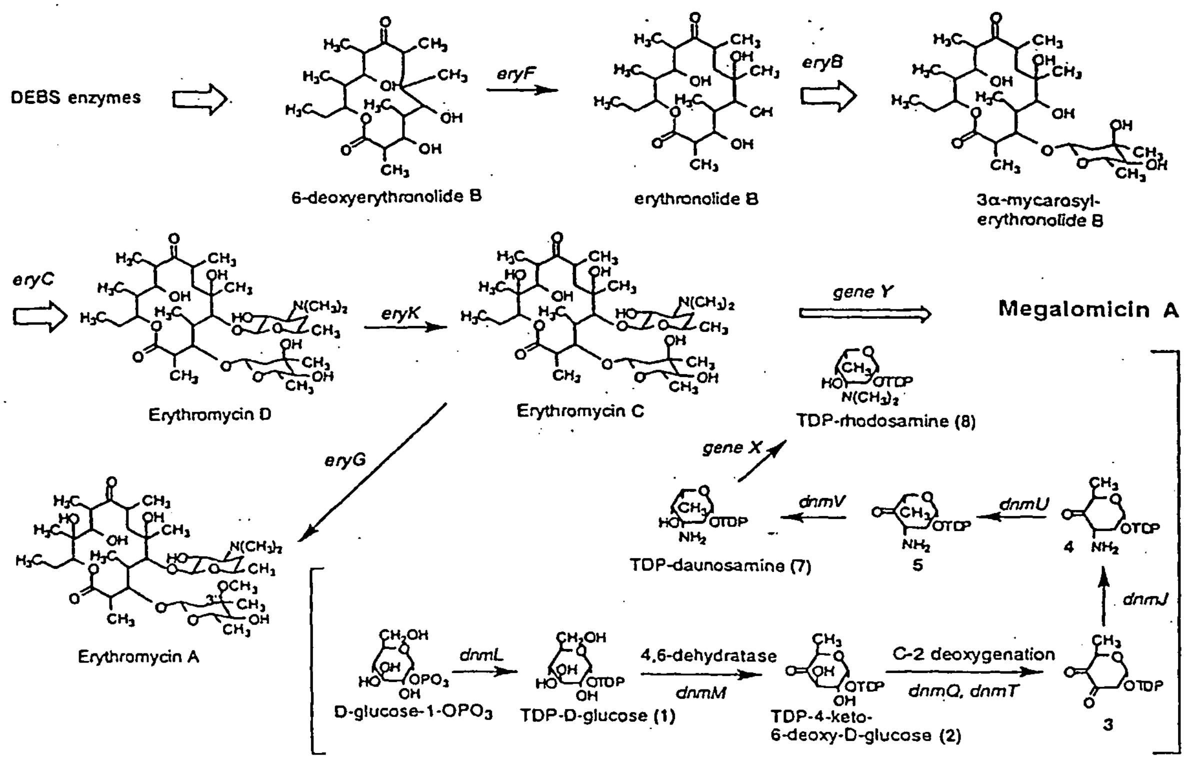
Structures of the Megalomicins and Azithromycin

Figure 3



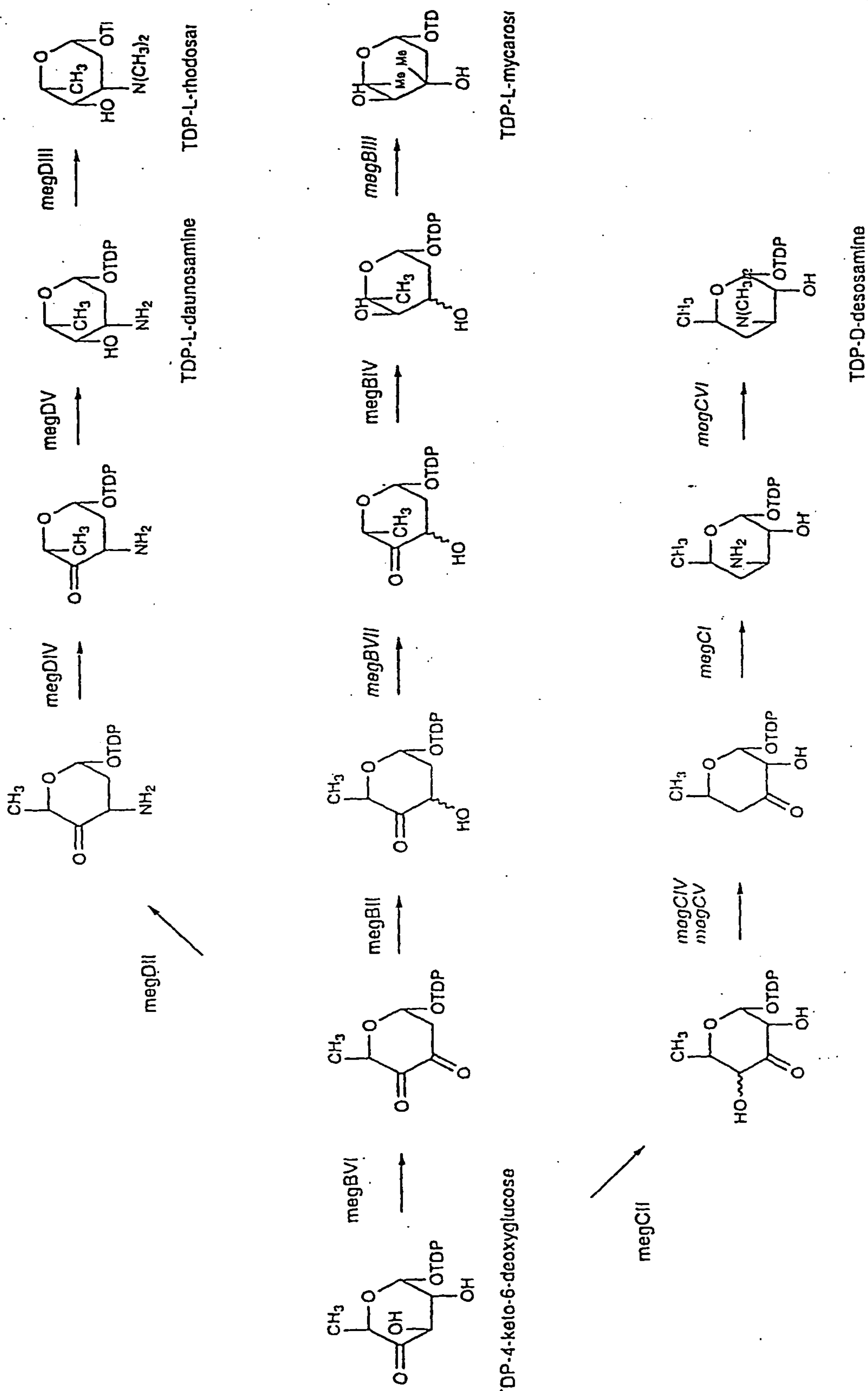
Biosynthesis of 6-Deoxyerythronolide B (6-dEB), the Aglycone of Erythromycin, by a Modular PKS

Figure 4



Erythromycin Biosynthetic Pathway and Megalomicin Biosynthesis

Figure 5



Glycoside Biosynthetic Genes

Figure 6

```
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VERSION
KEYWORDS    .
SOURCE      Micromonospora megalomicea.
  ORGANISM  Micromonospora megalomicea
            Unclassified.
REFERENCE   1  (bases 1 to 47981)
  AUTHORS   Volchegursky,Y., Hu,Z., Katz,L. and McDaniel,R.
  TITLE     Biosynthesis of the Anti-Parasitic Agent Megalomicin:
            Transformation of Erythromycin to Megalomicin in Saccharopolyspora
            erythraea
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 47981)
  AUTHORS   McDaniel,R. and Volchegursky,Y.
  TITLE     Direct Submission
  JOURNAL   Submitted (01-MAY-2000) Kosan Biosciences, Inc., 3828 Bay Center
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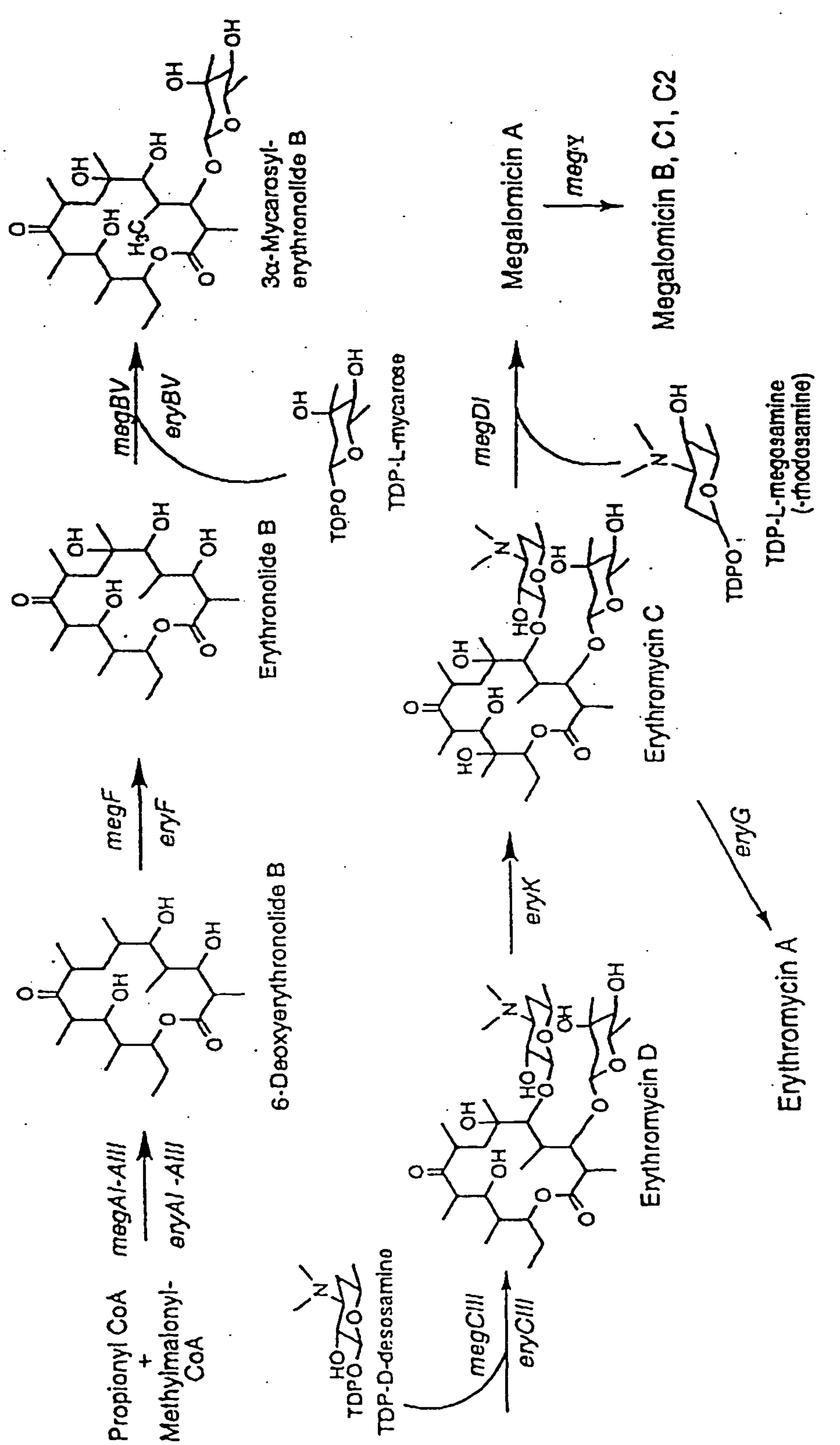

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megF
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Erythronolide B
megBV
eryBV
TDP-L-mycarose
3α-Mycarosyl-erythronolide B
TDP-D-desosamine
megCIII
eryCIII
Erythromycin D
eryK
Erythromycin C
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megY
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eryG
Erythromycin A

FIGURE 8

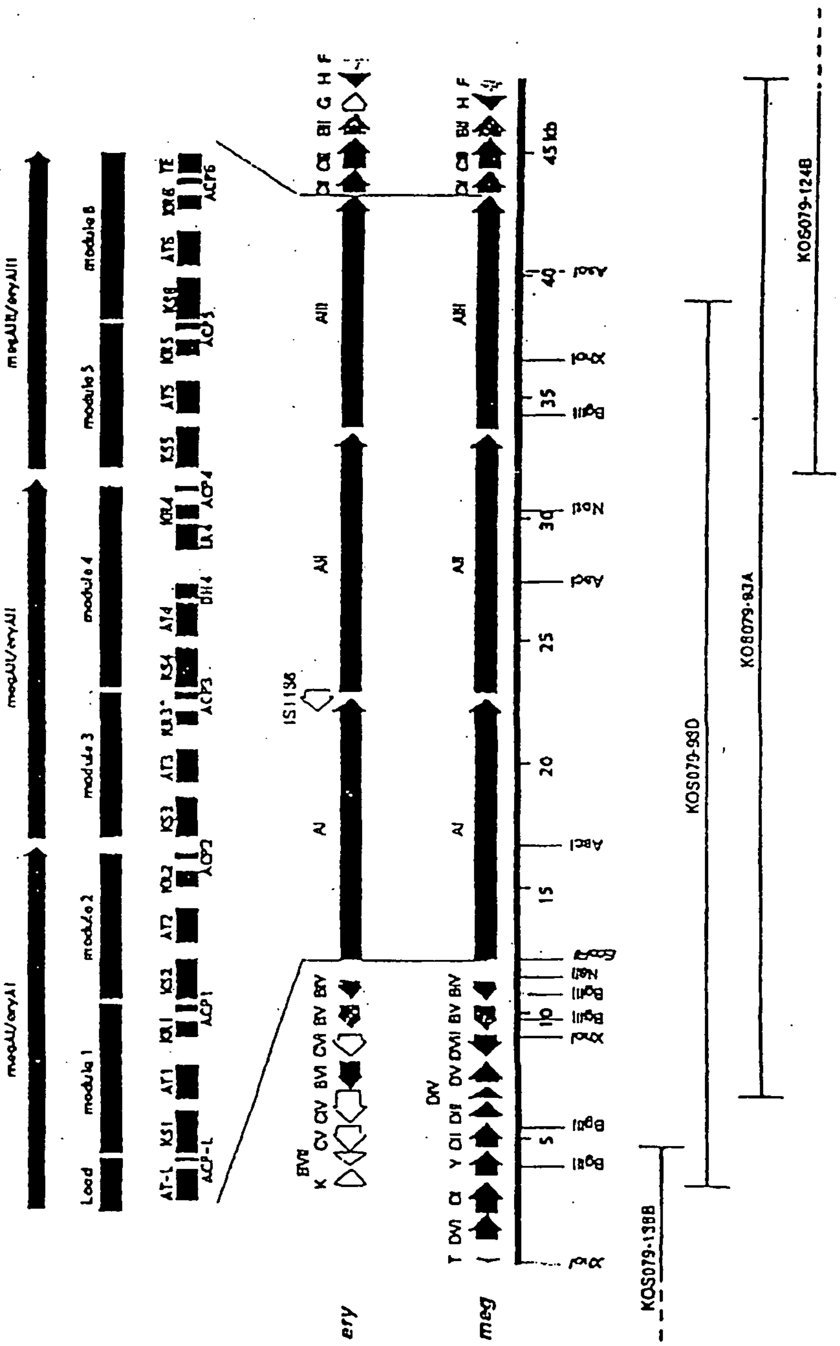
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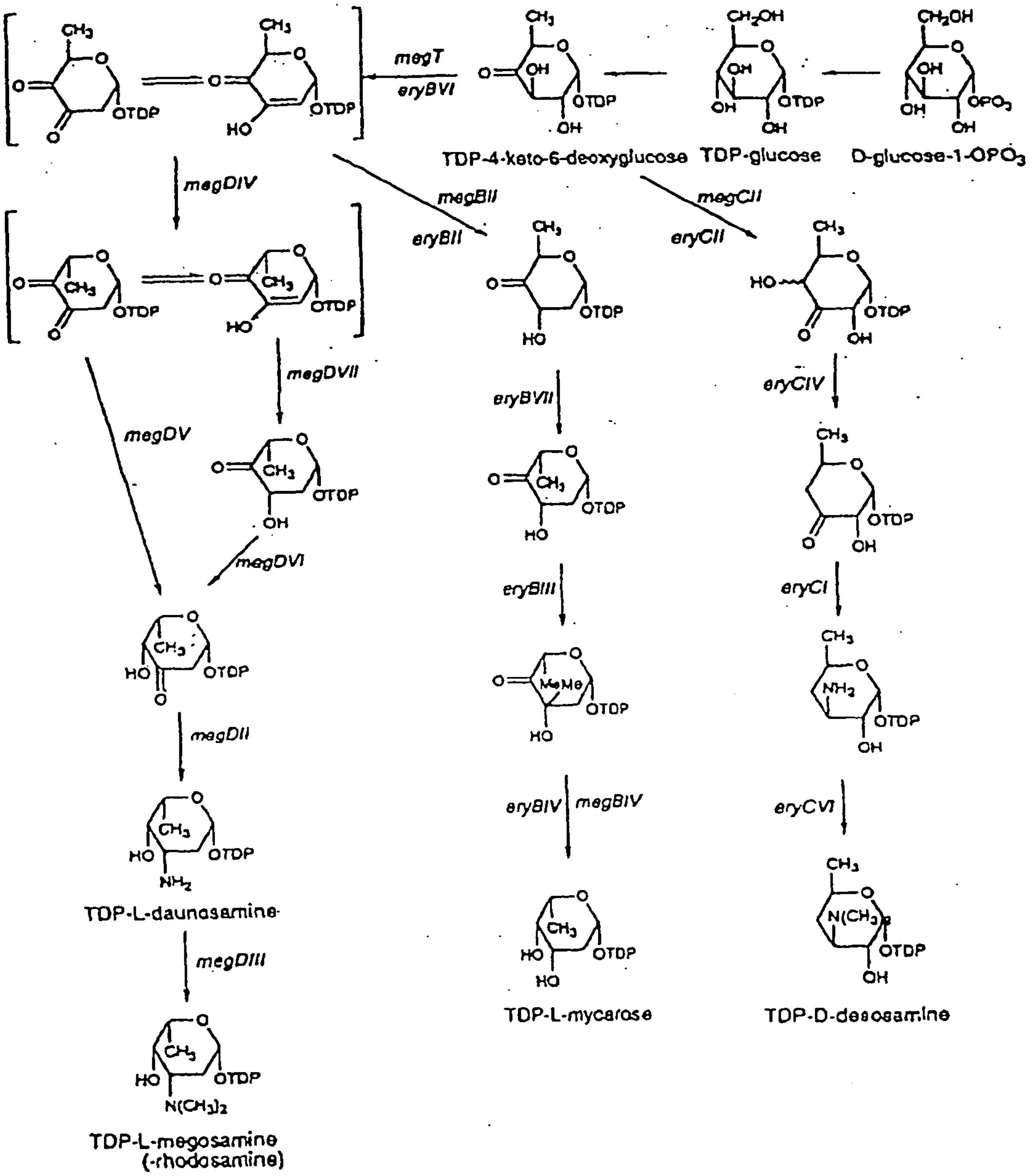
FIGURE 9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eryBIII
eryCIV
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megBIV
eryCVI
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FIGURE 10

SEQUENCE LISTING

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Genes and Uses Thereof

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SEQ ID NO: 2= translated amino acid sequence

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SEQ ID NO: 5= translated amino acid sequence

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<223> megAI; SEQ ID NO: 13= translated amino acid sequence

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<222> (13576)...(13791)
<223> megAI, ACP-L

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Arg Ala Ala Gly Arg Gly Pro Tyr Ala Ser Arg Ala Gly Thr Trp Val
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Val Gly Asp His Arg Thr Ala Ala Glu Val Leu Ala Asp Pro Gly Phe
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Thr His Gly Pro Pro Asp Ala Ala Arg Trp Met Gln Val Ala His Cys
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Cys Ala Arg Leu Val Thr Glu Leu Gly Ser Arg Phe Asp Leu Val Asn
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Asp Phe Ala Arg Glu Val Pro Val Leu Ala Leu Gly Thr Ala Pro Ala
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Glu Gln Ala Leu Thr Ala Leu Asp Glu Ile Asp Ala Val Thr Gly Gly
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Thr Ala Val Pro Val Gly Asp Asp Val Glu Leu Val Glu Trp His Ala
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His Ala Gly Gln Asp Ile Val Glu Tyr Met Arg Thr Leu Asp Trp Val
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Val Trp Glu Pro Leu Thr Phe Ala Ala Pro Ile Ala Ala Arg Val Thr
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Gly Thr Pro His Ala Arg Met Leu Trp Gly Pro Asp Val Ala Thr Arg
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Ala Arg Gln Ser Phe Leu Arg Leu Leu Ala His Gln Glu Val Glu His
                165                 170                 175
Arg Glu Asp Pro Leu Ala Glu Trp Phe Asp Trp Thr Leu Arg Arg Phe
            180                 185                 190
Gly Asp Asp Pro His Leu Ser Phe Asp Glu Glu Leu Val Leu Gly Gln
        195                 200                 205
Trp Thr Val Asp Pro Ile Pro Glu Pro Leu Arg Ile Asp Thr Gly Val
    210                 215                 220
Arg Thr Val Gly Met Arg Tyr Val Pro Tyr Asn Gly Pro Ser Val Val
225                 230                 235                 240
Pro Ala Trp Leu Leu Arg Glu Pro Glu Arg Arg Arg Val Cys Leu Thr
                245                 250                 255
Leu Gly Gly Ser Ser Arg Glu His Gly Ile Gly Gln Val Ser Ile Gly
            260                 265                 270
Glu Met Leu Asp Ala Ile Ala Asp Ile Asp Ala Glu Phe Val Ala Thr
        275                 280                 285
Phe Asp Asp Gln Gln Leu Val Gly Val Gly Ser Val Pro Ala Asn Val
    290                 295                 300
Arg Thr Ala Gly Phe Val Pro Met Asn Val Leu Leu Pro Thr Cys Ala
305                 310                 315                 320
Ala Thr Val His His Gly Gly Thr Gly Ser Trp Leu Thr Ala Ala Ile
                325                 330                 335
```

His Gly Val Pro Gln Ile Ile Leu Ser Asp Ala Asp Thr Glu Val His
340 345 350
Ala Lys Gln Leu Gln Asp Leu Gly Ala Gly Leu Ser Leu Pro Val Ala
355 360 365
Gly Met Thr Ala Glu His Leu Arg Gly Ala Ile Glu Arg Val Leu Asp
370 375 380
Glu Pro Ala Tyr Arg Leu Gly Ala Glu Arg Met Arg Asp Gly Met Arg
385 390 395 400
Thr Asp Pro Ser Pro Ala Gln Val Val Gly Ile Cys Gln Asp Leu Ala
405 410 415
Ala Asp Arg Ala Ala Arg Gly Arg Gln Pro Arg Arg Thr Ala Glu Pro
420 425 430
His Leu Pro Arg
435

<210> 5
<211> 390
<212> PRT
<213> Micromonospora megalomicea

<400> 5
Met Val Thr Ser Thr Asn Leu Asp Thr Thr Ala Arg Pro Ala Leu Asn
1 5 10 15
Ser Leu Thr Gly Met Arg Phe Val Ala Ala Phe Leu Val Phe Phe Thr
20 25 30
His Val Leu Ser Arg Leu Ile Pro Asn Ser Tyr Val Tyr Ala Asp Gly
35 40 45
Leu Asp Ala Phe Trp Gln Thr Thr Gly Arg Val Gly Val Ser Phe Phe
50 55 60
Phe Ile Leu Ser Gly Phe Val Leu Thr Trp Ser Ala Arg Ala Ser Asp
65 70 75 80
Ser Val Trp Ser Phe Trp Arg Arg Arg Val Cys Lys Leu Phe Pro Asn
85 90 95
His Leu Val Thr Ala Phe Ala Ala Val Val Leu Phe Leu Val Thr Gly
100 105 110
Gln Ala Val Ser Gly Glu Ala Leu Ile Pro Asn Leu Leu Leu Ile His
115 120 125
Ala Trp Phe Pro Ala Leu Glu Ile Ser Phe Gly Ile Asn Pro Val Ser
130 135 140
Trp Ser Leu Ala Cys Glu Ala Phe Phe Tyr Leu Cys Phe Pro Leu Phe
145 150 155 160
Leu Phe Trp Ile Ser Gly Ile Arg Pro Glu Arg Leu Trp Ala Trp Ala
165 170 175
Ala Val Val Phe Ala Ala Ile Trp Ala Val Pro Val Val Ala Asp Leu
180 185 190
Leu Leu Pro Ser Ser Pro Pro Leu Ile Pro Gly Leu Glu Tyr Ser Ala
195 200 205
Ile Gln Asp Trp Phe Leu Tyr Thr Phe Pro Ala Thr Arg Ser Leu Glu
210 215 220
Phe Ile Leu Gly Ile Ile Leu Ala Arg Ile Leu Ile Thr Gly Arg Trp
225 230 235 240
Ile Asn Val Gly Leu Leu Pro Ala Val Leu Leu Phe Pro Val Phe Phe
245 250 255
Val Ala Ser Leu Phe Leu Pro Gly Val Tyr Ala Ile Ser Ser Ser Met
260 265 270
Met Ile Leu Pro Leu Val Leu Ile Ile Ala Ser Gly Ala Thr Ala Asp
275 280 285
Leu Gln Gln Lys Arg Thr Phe Met Arg Asn Arg Val Met Val Trp Leu
290 295 300
Gly Asp Val Ser Phe Ala Leu Tyr Met Val His Phe Leu Val Ile Val
305 310 315 320

Tyr Gly Ala Asp Leu Leu Gly Phe Ser Gln Thr Glu Asp Ala Pro Leu
325 330 335
Gly Leu Ala Leu Phe Met Ile Ile Pro Phe Leu Ala Val Ser Leu Val
340 345 350
Leu Ser Trp Leu Leu Tyr Arg Phe Val Glu Leu Pro Val Met Arg Asn
355 360 365
Trp Ala Arg Pro Ala Ser Ala Arg Arg Lys Pro Ala Thr Glu Pro Glu
370 375 380
Gln Thr Pro Ser Arg Arg
385 390

<210> 6
<211> 374
<212> PRT
<213> Micromonospora megalomicea

<400> 6
Met Thr Thr Tyr Val Trp Ser Tyr Leu Leu Glu Tyr Glu Arg Glu Arg
1 5 10 15
Ala Asp Ile Leu Asp Ala Val Gln Lys Val Phe Ala Ser Gly Ser Leu
20 25 30
Ile Leu Gly Gln Ser Val Glu Asn Phe Glu Thr Glu Tyr Ala Arg Tyr
35 40 45
His Gly Ile Ala His Cys Val Gly Val Asp Asn Gly Thr Asn Ala Val
50 55 60
Lys Leu Ala Leu Glu Ser Val Gly Val Gly Arg Asp Asp Glu Val Val
65 70 75 80
Thr Val Ser Asn Thr Ala Ala Pro Thr Val Leu Ala Ile Asp Glu Ile
85 90 95
Gly Ala Arg Pro Val Phe Val Asp Val Arg Asp Glu Asp Tyr Leu Met
100 105 110
Asp Thr Asp Leu Val Glu Ala Ala Val Thr Pro Arg Thr Lys Ala Ile
115 120 125
Val Pro Val His Leu Tyr Gly Gln Cys Val Asp Met Thr Ala Leu Arg
130 135 140
Glu Leu Ala Asp Arg Arg Gly Leu Lys Leu Val Glu Asp Cys Ala Gln
145 150 155 160
Ala His Gly Ala Arg Arg Asp Gly Arg Leu Ala Gly Thr Met Ser Asp
165 170 175
Ala Ala Ala Phe Ser Phe Tyr Pro Thr Lys Val Leu Gly Ala Tyr Gly
180 185 190
Asp Gly Gly Ala Val Val Thr Asn Asp Asp Glu Thr Ala Arg Ala Leu
195 200 205
Arg Arg Leu Arg Tyr Tyr Gly Met Glu Glu Val Tyr Tyr Val Thr Arg
210 215 220
Thr Pro Gly His Asn Ser Arg Leu Asp Glu Val Gln Ala Glu Ile Leu
225 230 235 240
Arg Arg Lys Leu Thr Arg Leu Asp Ala Tyr Val Ala Gly Arg Arg Ala
245 250 255
Val Ala Gln Arg Tyr Val Asp Gly Leu Ala Asp Leu Gln Asp Ser His
260 265 270
Gly Leu Glu Leu Pro Val Val Thr Asp Gly Asn Glu His Val Phe Tyr
275 280 285
Val Tyr Val Val Arg His Pro Arg Arg Asp Glu Ile Ile Lys Arg Leu
290 295 300
Arg Asp Gly Tyr Asp Ile Ser Leu Asn Ile Ser Tyr Pro Trp Pro Val
305 310 315 320
His Thr Met Thr Gly Phe Ala His Leu Gly Val Ala Ser Gly Ser Leu
325 330 335
Pro Val Thr Glu Arg Leu Ala Gly Glu Ile Phe Ser Leu Pro Met Tyr
340 345 350

Pro Ser Leu Pro His Asp Leu Gln Asp Arg Val Ile Glu Ala Val Arg
355 360 365
Glu Val Ile Thr Gly Leu
370

<210> 7
<211> 257
<212> PRT
<213> Micromonospora megalomicea

<400> 7
Met Pro Asn Ser His Ser Thr Thr Ser Ser Thr Asp Val Ala Pro Tyr
1 5 10 15
Glu Arg Ala Asp Ile Tyr His Asp Phe Tyr His Gly Arg Gly Lys Gly
20 25 30
Tyr Arg Ala Glu Ala Asp Ala Leu Val Glu Val Ala Arg Lys His Thr
35 40 45
Pro Gln Ala Ala Thr Leu Leu Asp Val Ala Cys Gly Thr Gly Ser His
50 55 60
Leu Val Glu Leu Ala Asp Ser Phe Arg Glu Val Val Gly Val Asp Leu
65 70 75 80
Ser Ala Ala Met Leu Ala Thr Ala Ala Arg Asn Asp Pro Gly Arg Glu
85 90 95
Leu His Gln Gly Asp Met Arg Asp Phe Ser Leu Asp Arg Arg Phe Asp
100 105 110
Val Val Thr Cys Met Phe Ser Ser Thr Gly Tyr Leu Val Asp Glu Ala
115 120 125
Glu Leu Asp Arg Ala Val Ala Asn Leu Ala Gly His Leu Ala Pro Gly
130 135 140
Gly Thr Leu Val Val Glu Pro Trp Trp Phe Pro Glu Thr Phe Arg Pro
145 150 155 160
Gly Trp Val Gly Ala Asp Leu Val Thr Ser Gly Asp Arg Arg Ile Ser
165 170 175
Arg Met Ser His Thr Val Pro Ala Gly Leu Pro Asp Arg Thr Ala Ser
180 185 190
Arg Met Thr Ile His Tyr Thr Val Gly Ser Pro Glu Ala Gly Ile Glu
195 200 205
His Phe Thr Glu Val His Val Met Thr Leu Phe Ala Arg Ala Ala Tyr
210 215 220
Glu Gln Ala Phe Gln Arg Ala Gly Leu Ser Cys Ser Tyr Val Gly His
225 230 235 240
Asp Leu Phe Ser Pro Gly Leu Phe Val Gly Val Ala Ala Glu Pro Gly
245 250 255
Arg

<210> 8
<211> 201
<212> PRT
<213> Micromonospora megalomicea

<400> 8
Met Arg Val Glu Glu Leu Gly Ile Glu Gly Val Phe Thr Phe Thr Pro
1 5 10 15
Gln Thr Phe Ala Asp Glu Arg Gly Val Phe Gly Thr Ala Tyr Gln Glu
20 25 30
Asp Val Phe Val Ala Ala Leu Gly Arg Pro Leu Phe Pro Val Ala Gln
35 40 45
Val Ser Thr Thr Arg Ser Arg Arg Gly Val Val Arg Gly Val His Phe
50 55 60
Thr Thr Met Pro Gly Ser Met Ala Lys Tyr Val Tyr Cys Ala Arg Gly

65 70 75 80
Arg Ala Met Asp Phe Ala Val Asp Ile Arg Pro Gly Ser Pro Thr Phe
85 90 95
Gly Arg Ala Glu Pro Val Glu Leu Ser Ala Glu Ser Met Val Gly Leu
100 105 110
Tyr Leu Pro Val Gly Met Gly His Leu Phe Val Ser Leu Glu Asp Asp
115 120 125
Thr Thr Leu Val Tyr Leu Met Ser Ala Gly Tyr Val Pro Asp Lys Glu
130 135 140
Arg Ala Val His Pro Leu Asp Pro Glu Leu Ala Leu Pro Ile Pro Ala
145 150 155 160
Asp Leu Asp Leu Val Met Ser Glu Arg Asp Arg Val Ala Pro Thr Leu
165 170 175
Arg Glu Ala Arg Asp Gln Gly Ile Leu Pro Asp Tyr Ala Ala Cys Arg
180 185 190
Ala Ala Ala His Arg Val Val Arg Thr
195 200

<210> 9
<211> 328
<212> PRT
<213> Micromonospora megalomicea

<400> 9
Met Val Val Leu Gly Ala Ser Gly Phe Leu Gly Ser Ala Val Thr His
1 5 10 15
Ala Leu Ala Asp Leu Pro Val Arg Val Arg Leu Val Ala Arg Arg Glu
20 25 30
Val Val Val Pro Ser Gly Ala Val Ala Asp Tyr Glu Thr His Arg Val
35 40 45
Asp Leu Thr Glu Pro Gly Ala Leu Ala Glu Val Val Ala Asp Ala Arg
50 55 60
Ala Val Phe Pro Phe Ala Ala Gln Ile Arg Gly Thr Ser Gly Trp Arg
65 70 75 80
Ile Ser Glu Asp Asp Val Val Ala Glu Arg Thr Asn Val Gly Leu Val
85 90 95
Arg Asp Leu Ile Ala Val Leu Ser Arg Ser Pro His Ala Pro Val Val
100 105 110
Val Phe Pro Gly Ser Asn Thr Gln Val Gly Arg Val Thr Ala Gly Arg
115 120 125
Val Ile Asp Gly Ser Glu Gln Asp His Pro Glu Gly Val Tyr Asp Arg
130 135 140
Gln Lys His Thr Gly Glu Gln Leu Leu Lys Glu Ala Thr Ala Ala Gly
145 150 155 160
Ala Ile Arg Ala Thr Ser Leu Arg Leu Pro Pro Val Phe Gly Val Pro
165 170 175
Ala Ala Gly Thr Ala Asp Asp Arg Gly Val Val Ser Thr Met Ile Arg
180 185 190
Arg Ala Leu Thr Gly Gln Pro Leu Thr Met Trp His Asp Gly Thr Val
195 200 205
Arg Arg Glu Leu Leu Tyr Val Thr Asp Ala Ala Arg Ala Phe Val Thr
210 215 220
Ala Leu Asp His Ala Asp Ala Leu Ala Gly Arg His Phe Leu Leu Gly
225 230 235 240
Thr Gly Arg Ser Trp Pro Leu Gly Glu Val Phe Gln Ala Val Ser Arg
245 250 255
Ser Val Ala Arg His Thr Gly Glu Asp Pro Val Pro Val Val Ser Val
260 265 270
Pro Pro Pro Ala His Met Asp Pro Ser Asp Leu Arg Ser Val Glu Val
275 280 285
Asp Pro Ala Arg Phe Thr Ala Val Thr Gly Trp Arg Ala Thr Val Thr

290 295 300
Met Ala Glu Ala Val Asp Arg Thr Val Ala Ala Leu Ala Pro Arg Arg
305 310 315 320
Ala Ala Ala Pro Ser Glu Pro Ser
325

<210> 10
<211> 330
<212> PRT
<213> Micromonospora megalomicea

<400> 10
Met Gly Thr Thr Gly Ala Gly Ser Ala Arg Val Arg Val Gly Arg Ser
1 5 10 15
Ala Leu His Thr Ser Arg Leu Trp Leu Gly Thr Val Asn Phe Ser Gly
20 25 30
Arg Val Thr Asp Asp Asp Ala Leu Arg Leu Met Asp His Ala Leu Glu
35 40 45
Arg Gly Val Asn Cys Ile Asp Thr Ala Asp Ile Tyr Gly Trp Arg Leu
50 55 60
Tyr Lys Gly His Thr Glu Glu Leu Val Gly Arg Trp Phe Ala Gln Gly
65 70 75 80
Gly Gly Arg Arg Glu Glu Thr Val Leu Ala Thr Lys Val Gly Ser Glu
85 90 95
Met Ser Glu Arg Val Asn Asp Gly Gly Leu Ser Ala Arg His Ile Val
100 105 110
Ala Ala Cys Glu Asn Ser Leu Arg Arg Leu Gly Val Asp His Ile Asp
115 120 125
Ile Tyr Gln Thr His His Ile Asp Arg Ala Ala Pro Trp Asp Glu Val
130 135 140
Trp Gln Ala Ala Glu His Leu Val Gly Ser Gly Lys Val Gly Tyr Val
145 150 155 160
Gly Ser Ser Asn Leu Ala Gly Trp His Ile Ala Ala Ala Gln Glu Ser
165 170 175
Ala Ala Arg Arg Asn Leu Leu Gly Met Ile Ser His Gln Cys Leu Tyr
180 185 190
Asn Leu Ala Val Arg His Pro Glu Leu Asp Val Leu Pro Ala Ala Gln
195 200 205
Ala Tyr Gly Val Gly Val Phe Ala Trp Ser Pro Leu His Gly Gly Leu
210 215 220
Leu Ser Gly Val Leu Glu Lys Leu Ala Ala Gly Thr Ala Val Lys Ser
225 230 235 240
Ala Gln Gly Arg Ala Gln Val Leu Leu Pro Ala Val Arg Pro Leu Val
245 250 255
Glu Ala Tyr Glu Asp Tyr Cys Arg Arg Leu Gly Ala Asp Pro Ala Glu
260 265 270
Val Gly Leu Ala Trp Val Leu Ser Arg Pro Gly Ile Leu Gly Ala Val
275 280 285
Ile Gly Pro Arg Thr Pro Glu Gln Leu Asp Ser Ala Leu Arg Ala Ala
290 295 300
Glu Leu Thr Leu Gly Glu Glu Glu Leu Arg Glu Leu Glu Ala Ile Phe
305 310 315 320
Pro Ala Pro Ala Val Asp Gly Pro Val Pro
325 330

<210> 11
<211> 417
<212> PRT
<213> Micromonospora megalomicea

<400> 11

Met Arg Val Leu Leu Thr Ser Phe Ala His Arg Thr His Phe Gln Gly
1 5 10 15
Leu Val Pro Leu Ala Trp Ala Leu His Thr Ala Gly His Asp Val Arg
20 25 30
Val Ala Ser Gln Pro Glu Leu Thr Asp Val Val Val Gly Ala Gly Leu
35 40 45
Thr Ser Val Pro Leu Gly Ser Asp His Arg Leu Phe Asp Ile Ser Pro
50 55 60
Glu Ala Ala Ala Gln Val His Arg Tyr Thr Thr Asp Leu Asp Phe Ala
65 70 75 80
Arg Arg Gly Pro Glu Leu Arg Ser Trp Glu Phe Leu His Gly Ile Glu
85 90 95
Glu Ala Thr Ser Arg Phe Val Phe Pro Val Val Asn Asn Asp Ser Phe
100 105 110
Val Asp Glu Leu Val Glu Phe Ala Met Asp Trp Arg Pro Asp Leu Val
115 120 125
Leu Trp Glu Pro Phe Thr Phe Ala Gly Ala Val Ala Ala Lys Ala Cys
130 135 140
Gly Ala Ala His Ala Arg Leu Leu Trp Gly Ser Asp Leu Thr Gly Tyr
145 150 155 160
Phe Arg Ser Arg Ser Gln Asp Leu Arg Gly Gln Arg Pro Ala Asp Asp
165 170 175
Arg Pro Asp Pro Leu Gly Gly Trp Leu Thr Glu Val Ala Gly Arg Phe
180 185 190
Gly Leu Asp Tyr Ser Glu Asp Leu Ala Val Gly Gln Trp Ser Val Asp
195 200 205
Gln Leu Pro Glu Ser Phe Arg Leu Glu Thr Gly Leu Glu Ser Val His
210 215 220
Thr Arg Thr Leu Pro Tyr Asn Gly Ser Ser Val Val Pro Gln Trp Leu
225 230 235 240
Arg Thr Ser Asp Gly Val Arg Arg Val Cys Phe Thr Gly Gly Tyr Ser
245 250 255
Ala Leu Gly Ile Thr Ser Asn Pro Gln Glu Phe Leu Arg Thr Leu Ala
260 265 270
Thr Leu Ala Arg Phe Asp Gly Glu Ile Val Val Thr Arg Ser Gly Leu
275 280 285
Asp Pro Ala Ser Val Pro Asp Asn Val Arg Leu Val Asp Phe Val Pro
290 295 300
Met Asn Ile Leu Leu Pro Gly Cys Ala Ala Val Ile His His Gly Gly
305 310 315 320
Ala Gly Ser Trp Ala Thr Ala Leu His His Gly Val Pro Gln Ile Ser
325 330 335
Val Ala His Glu Trp Asp Cys Val Leu Arg Gly Gln Arg Thr Ala Glu
340 345 350
Leu Gly Ala Gly Val Phe Leu Arg Pro Asp Glu Val Asp Ala Asp Thr
355 360 365
Leu Trp Gln Ala Leu Ala Thr Val Val Glu Asp Arg Ser His Ala Glu
370 375 380
Asn Ala Glu Lys Leu Arg Gln Glu Ala Leu Ala Ala Pro Thr Pro Ala
385 390 395 400
Glu Val Val Pro Val Leu Glu Ala Leu Ala His Gln His Arg Ala Asp
405 410 415
Arg

<210> 12
<211> 313
<212> PRT
<213> Micromonospora megalomicea

<400> 12

Met Thr Arg His Val Thr Leu Leu Gly Val Ser Gly Phe Val Gly Ser
1 5 10 15
Ala Leu Leu Arg Glu Phe Thr Thr His Pro Leu Arg Leu Arg Ala Val
20 25 30
Ala Arg Thr Gly Ser Arg Asp Gln Pro Pro Gly Ser Ala Gly Ile Glu
35 40 45
His Leu Arg Val Asp Leu Leu Glu Pro Gly Arg Val Ala Gln Val Val
50 55 60
Ala Asp Thr Asp Val Val Val His Leu Val Ala Tyr Ala Ala Gly Gly
65 70 75 80
Ser Thr Trp Arg Ser Ala Ala Thr Val Pro Glu Ala Glu Arg Val Asn
85 90 95
Ala Gly Ile Met Arg Asp Leu Val Ala Ala Leu Arg Ala Arg Pro Gly
100 105 110
Pro Ala Pro Val Leu Leu Phe Ala Ser Thr Thr Gln Ala Ala Asn Pro
115 120 125
Ala Ala Pro Ser Arg Tyr Ala Gln His Lys Ile Glu Ala Glu Arg Ile
130 135 140
Leu Arg Gln Ala Thr Glu Asp Gly Val Val Asp Gly Val Ile Leu Arg
145 150 155 160
Leu Pro Ala Ile Tyr Gly His Ser Gly Pro Ser Gly Gln Thr Gly Arg
165 170 175
Gly Val Val Thr Ala Met Ile Arg Arg Ala Leu Ala Gly Glu Pro Ile
180 185 190
Thr Met Trp His Glu Gly Ser Val Arg Arg Asn Leu Leu His Val Glu
195 200 205
Asp Val Ala Thr Ala Phe Thr Ala Ala Leu His Asn His Glu Ala Leu
210 215 220
Val Gly Asp Val Trp Thr Pro Ser Ala Asp Glu Ala Arg Pro Leu Gly
225 230 235 240
Glu Ile Phe Glu Thr Val Ala Ala Ser Val Ala Arg Gln Thr Gly Asn
245 250 255
Pro Ala Val Pro Val Val Ser Val Pro Pro Pro Glu Asn Ala Glu Ala
260 265 270
Asn Asp Phe Arg Ser Asp Asp Phe Asp Ser Thr Glu Phe Arg Thr Leu
275 280 285
Thr Gly Trp His Pro Arg Val Pro Leu Ala Glu Gly Ile Asp Arg Thr
290 295 300
Val Ala Ala Leu Ile Ser Thr Lys Glu
305 310

<210> 13
<211> 3546
<212> PRT
<213> Micromonospora megalomicea

<400> 13
Met Val Asp Val Pro Asp Leu Leu Gly Thr Arg Thr Pro His Pro Gly
1 5 10 15
Pro Leu Pro Phe Pro Trp Pro Leu Cys Gly His Asn Glu Pro Glu Leu
20 25 30
Arg Ala Arg Ala Arg Gln Leu His Ala Tyr Leu Glu Gly Ile Ser Glu
35 40 45
Asp Asp Val Val Ala Val Gly Ala Ala Leu Ala Arg Glu Thr Arg Ala
50 55 60
Gln Asp Gly Pro His Arg Ala Val Val Val Ala Ser Ser Val Thr Glu
65 70 75 80
Leu Thr Ala Ala Leu Ala Ala Leu Ala Gln Gly Arg Pro His Pro Ser
85 90 95
Val Val Arg Gly Val Ala Arg Pro Thr Ala Pro Val Val Phe Val Leu
100 105 110

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Pro Gly Gln Gly Ala Gln Trp Pro Gly Met Ala Thr Arg Leu Leu Ala
        115                 120                 125
Glu Ser Pro Val Phe Ala Ala Ala Met Arg Ala Cys Glu Arg Ala Phe
    130                 135                 140
Asp Glu Val Thr Asp Trp Ser Leu Thr Glu Val Leu Asp Ser Pro Glu
145                 150                 155                 160
His Leu Arg Arg Val Glu Val Val Gln Pro Ala Leu Phe Ala Val Gln
                165                 170                 175
Thr Ser Leu Ala Ala Leu Trp Arg Ser Phe Gly Val Arg Pro Asp Ala
            180                 185                 190
Val Leu Gly His Ser Ile Gly Glu Leu Ala Ala Ala Glu Val Cys Gly
        195                 200                 205
Ala Val Asp Val Glu Ala Ala Ala Arg Ala Ala Ala Leu Trp Ser Arg
    210                 215                 220
Glu Met Val Pro Leu Val Gly Arg Gly Asp Met Ala Ala Val Ala Leu
225                 230                 235                 240
Ser Pro Ala Glu Leu Ala Ala Arg Val Glu Arg Trp Asp Asp Asp Val
                245                 250                 255
Val Pro Ala Gly Val Asn Gly Pro Arg Ser Val Leu Leu Thr Gly Ala
            260                 265                 270
Pro Glu Pro Ile Ala Arg Arg Val Ala Glu Leu Ala Ala Gln Gly Val
        275                 280                 285
Arg Ala Gln Val Val Asn Val Ser Met Ala Ala His Ser Ala Gln Val
    290                 295                 300
Asp Ala Val Ala Glu Gly Met Arg Ser Ala Leu Thr Trp Phe Ala Pro
305                 310                 315                 320
Gly Asp Ser Asp Val Pro Tyr Tyr Ala Gly Leu Thr Gly Gly Arg Leu
                325                 330                 335
Asp Thr Arg Glu Leu Gly Ala Asp His Trp Pro Arg Ser Phe Arg Leu
            340                 345                 350
Pro Val Arg Phe Asp Glu Ala Thr Arg Ala Val Leu Glu Leu Gln Pro
        355                 360                 365
Gly Thr Phe Ile Glu Ser Ser Pro His Pro Val Leu Ala Ala Ser Leu
    370                 375                 380
Gln Gln Thr Leu Asp Glu Val Gly Ser Pro Ala Ala Ile Val Pro Thr
385                 390                 395                 400
Leu Gln Arg Asp Gln Gly Gly Leu Arg Arg Phe Leu Leu Ala Val Ala
                405                 410                 415
Gln Ala Tyr Thr Gly Gly Val Thr Val Asp Trp Thr Ala Ala Tyr Pro
            420                 425                 430
Gly Val Thr Pro Gly His Leu Pro Ser Ala Val Ala Val Glu Thr Asp
        435                 440                 445
Glu Gly Pro Ser Thr Glu Phe Asp Trp Ala Ala Pro Asp His Val Leu
    450                 455                 460
Arg Ala Arg Leu Leu Glu Ile Val Gly Ala Glu Thr Ala Ala Leu Ala
465                 470                 475                 480
Gly Arg Glu Val Asp Ala Arg Ala Thr Phe Arg Glu Leu Gly Leu Asp
                485                 490                 495
Ser Val Leu Ala Val Gln Leu Arg Thr Arg Leu Ala Thr Ala Thr Gly
            500                 505                 510
Arg Asp Leu His Ile Ala Met Leu Tyr Asp His Pro Thr Pro His Ala
        515                 520                 525
Leu Thr Glu Ala Leu Leu Arg Gly Pro Gln Glu Glu Pro Gly Arg Gly
    530                 535                 540
Glu Glu Thr Ala His Pro Thr Glu Ala Glu Pro Asp Glu Pro Val Ala
545                 550                 555                 560
Val Val Ala Met Ala Cys Arg Leu Pro Gly Gly Val Thr Ser Pro Glu
                565                 570                 575
Glu Phe Trp Glu Leu Leu Ala Glu Gly Arg Asp Ala Val Gly Gly Leu
            580                 585                 590
Pro Thr Asp Arg Gly Trp Asp Leu Asp Ser Leu Phe His Pro Asp Pro
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595 600 605
Thr Arg Ser Gly Thr Ala His Gln Arg Ala Gly Gly Phe Leu Thr Gly
610 615 620
Ala Thr Ser Phe Asp Ala Ala Phe Phe Gly Leu Ser Pro Arg Glu Ala
625 630 635 640
Leu Ala Val Glu Pro Gln Gln Arg Ile Thr Leu Glu Leu Ser Trp Glu
645 650 655
Val Leu Glu Arg Ala Gly Ile Pro Pro Thr Ser Leu Arg Thr Ser Arg
660 665 670
Thr Gly Val Phe Val Gly Leu Ile Pro Gln Glu Tyr Gly Pro Arg Leu
675 680 685
Ala Glu Gly Gly Glu Gly Val Glu Gly Tyr Leu Met Thr Gly Thr Thr
690 695 700
Thr Ser Val Ala Ser Gly Arg Val Ala Tyr Thr Leu Gly Leu Glu Gly
705 710 715 720
Pro Ala Ile Ser Val Asp Thr Ala Cys Ser Ser Ser Leu Val Ala Val
725 730 735
His Leu Ala Cys Gln Ser Leu Arg Arg Gly Glu Ser Thr Met Ala Leu
740 745 750
Ala Gly Gly Val Thr Val Met Pro Thr Pro Gly Met Leu Val Asp Phe
755 760 765
Ser Arg Met Asn Ser Leu Ala Pro Asp Gly Arg Ser Lys Ala Phe Ser
770 775 780
Ala Ala Ala Asp Gly Phe Gly Met Ala Glu Gly Ala Gly Met Leu Leu
785 790 795 800
Leu Glu Arg Leu Ser Asp Ala Arg Arg His Gly His Pro Val Leu Ala
805 810 815
Val Ile Arg Gly Thr Ala Val Asn Ser Asp Gly Ala Ser Asn Gly Leu
820 825 830
Ser Ala Pro Asn Gly Arg Ala Gln Val Arg Val Ile Arg Gln Ala Leu
835 840 845
Ala Glu Ser Gly Leu Thr Pro His Thr Val Asp Val Val Glu Thr His
850 855 860
Gly Thr Gly Thr Arg Leu Gly Asp Pro Ile Glu Ala Arg Ala Leu Ser
865 870 875 880
Asp Ala Tyr Gly Gly Asp Arg Glu His Pro Leu Arg Ile Gly Ser Val
885 890 895
Lys Ser Asn Ile Gly His Thr Gln Ala Ala Ala Gly Val Ala Gly Leu
900 905 910
Ile Lys Leu Val Leu Ala Met Gln Ala Gly Val Leu Pro Arg Thr Leu
915 920 925
His Ala Asp Glu Pro Ser Pro Glu Ile Asp Trp Ser Ser Gly Ala Ile
930 935 940
Ser Leu Leu Gln Glu Pro Ala Ala Trp Pro Ala Gly Glu Arg Pro Arg
945 950 955 960
Arg Ala Gly Val Ser Ser Phe Gly Ile Ser Gly Thr Asn Ala His Ala
965 970 975
Ile Ile Glu Glu Ala Pro Pro Thr Gly Asp Asp Thr Arg Pro Asp Arg
980 985 990
Met Gly Pro Val Val Pro Trp Val Leu Ser Ala Ser Thr Gly Glu Ala
995 1000 1005
Leu Arg Ala Arg Ala Ala Arg Leu Ala Gly His Leu Arg Glu His Pro
1010 1015 1020
Asp Gln Asp Leu Asp Asp Val Ala Tyr Ser Leu Ala Thr Gly Arg Ala
1025 1030 1035 1040
Ala Leu Ala Tyr Arg Ser Gly Phe Val Pro Ala Asp Ala Ser Thr Ala
1045 1050 1055
Leu Arg Ile Leu Asp Glu Leu Ala Ala Gly Gly Ser Gly Asp Ala Val
1060 1065 1070
Thr Gly Thr Ala Arg Ala Pro Gln Arg Val Val Phe Val Phe Pro Gly
1075 1080 1085

Gln Gly Trp Gln Trp Ala Gly Met Ala Val Asp Leu Leu Asp Gly Asp
1090 1095 1100
Pro Val Phe Ala Ser Val Leu Arg Glu Cys Ala Asp Ala Leu Glu Pro
1105 1110 1115 1120
Tyr Leu Asp Phe Glu Ile Val Pro Phe Leu Arg Ala Glu Ala Gln Arg
1125 1130 1135
Arg Thr Pro Asp His Thr Leu Ser Thr Asp Arg Val Asp Val Val Gln
1140 1145 1150
Pro Val Leu Phe Ala Val Met Val Ser Leu Ala Ala Arg Trp Arg Ala
1155 1160 1165
Tyr Gly Val Glu Pro Ala Ala Val Ile Gly His Ser Gln Gly Glu Ile
1170 1175 1180
Ala Ala Ala Cys Val Ala Gly Ala Leu Ser Leu Asp Asp Ala Ala Arg
1185 1190 1195 1200
Ala Val Ala Leu Arg Ser Arg Val Ile Ala Thr Met Pro Gly Asn Gly
1205 1210 1215
Ala Met Ala Ser Ile Ala Ala Ser Val Asp Glu Val Ala Ala Arg Ile
1220 1225 1230
Asp Gly Arg Val Glu Ile Ala Ala Val Asn Gly Pro Arg Ala Val Val
1235 1240 1245
Val Ser Gly Asp Arg Asp Asp Leu Asp Arg Leu Val Ala Ser Cys Thr
1250 1255 1260
Val Glu Gly Val Arg Ala Lys Arg Leu Pro Val Asp Tyr Ala Ser His
1265 1270 1275 1280
Ser Ser His Val Glu Ala Val Arg Asp Ala Leu His Ala Glu Leu Gly
1285 1290 1295
Glu Phe Arg Pro Leu Pro Gly Phe Val Pro Phe Tyr Ser Thr Val Thr
1300 1305 1310
Gly Arg Trp Val Glu Pro Ala Glu Leu Asp Ala Gly Tyr Trp Phe Arg
1315 1320 1325
Asn Leu Arg His Arg Val Arg Phe Ala Asp Ala Val Arg Ser Leu Ala
1330 1335 1340
Asp Gln Gly Tyr Thr Thr Phe Leu Glu Val Ser Ala His Pro Val Leu
1345 1350 1355 1360
Thr Thr Ala Ile Glu Glu Ile Gly Glu Asp Arg Gly Gly Asp Leu Val
1365 1370 1375
Ala Val His Ser Leu Arg Arg Gly Ala Gly Gly Pro Val Asp Phe Gly
1380 1385 1390
Ser Ala Leu Ala Arg Ala Phe Val Ala Gly Val Ala Val Asp Trp Glu
1395 1400 1405
Ser Ala Tyr Gln Gly Ala Gly Ala Arg Arg Val Pro Leu Pro Thr Tyr
1410 1415 1420
Pro Phe Gln Arg Glu Arg Phe Trp Leu Glu Pro Asn Pro Ala Arg Arg
1425 1430 1435 1440
Val Ala Asp Ser Asp Asp Val Ser Ser Leu Arg Tyr Arg Ile Glu Trp
1445 1450 1455
His Pro Thr Asp Pro Gly Glu Pro Gly Arg Leu Asp Gly Thr Trp Leu
1460 1465 1470
Leu Ala Thr Tyr Pro Gly Arg Ala Asp Asp Arg Val Glu Ala Ala Arg
1475 1480 1485
Gln Ala Leu Glu Ser Ala Gly Ala Arg Val Glu Asp Leu Val Val Glu
1490 1495 1500
Pro Arg Thr Gly Arg Val Asp Leu Val Arg Arg Leu Asp Ala Val Gly
1505 1510 1515 1520
Pro Val Ala Gly Val Leu Cys Leu Phe Ala Val Ala Glu Pro Ala Ala
1525 1530 1535
Glu His Ser Pro Leu Ala Val Thr Ser Leu Ser Asp Thr Leu Asp Leu
1540 1545 1550
Thr Gln Ala Val Ala Gly Ser Gly Arg Glu Cys Pro Ile Trp Val Val
1555 1560 1565
Thr Glu Asn Ala Val Ala Val Gly Pro Phe Glu Arg Leu Arg Asp Pro

1570 1575 1580
Ala His Gly Ala Leu Trp Ala Leu Gly Arg Val Val Ala Leu Glu Asn
1585 1590 1595 1600
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Arg Ala Leu Leu Ala Thr Val Asp Asp Glu His Pro Leu Ser Ala Val
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Phe His Val Ala Ala Thr Leu Asp Asp Gly Thr Val Glu Thr Leu Thr
1745 1750 1755 1760
Gly Asp Arg Ile Glu Arg Ala Asn Arg Ala Lys Val Leu Gly Ala Arg
1765 1770 1775
Asn Leu His Glu Leu Thr Arg Asp Ala Asp Leu Asp Ala Phe Val Leu
1780 1785 1790
Phe Ser Ser Ser Thr Ala Ala Phe Gly Ala Pro Gly Leu Gly Gly Tyr
1795 1800 1805
Val Pro Gly Asn Ala Tyr Leu Asp Gly Leu Ala Gln Gln Arg Arg Ser
1810 1815 1820
Glu Gly Leu Pro Ala Thr Ser Val Ala Trp Gly Thr Trp Ala Gly Ser
1825 1830 1835 1840
Gly Met Ala Glu Gly Pro Val Ala Asp Arg Phe Arg Arg His Gly Val
1845 1850 1855
Met Glu Met His Pro Asp Gln Ala Val Glu Gly Leu Arg Val Ala Leu
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Val Gln Gly Glu Val Ala Pro Ile Val Val Asp Ile Arg Trp Asp Arg
1875 1880 1885
Phe Leu Leu Ala Tyr Thr Ala Gln Arg Pro Thr Arg Leu Phe Asp Thr
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Leu Asp Glu Ala Arg Arg Ala Ala Pro Gly Pro Asp Ala Gly Pro Gly
1905 1910 1915 1920
Val Ala Ala Leu Ala Gly Leu Pro Val Gly Glu Arg Glu Lys Ala Val
1925 1930 1935
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1940 1945 1950
Ala Glu Gln Val Pro Val Asp Arg Ala Phe Ala Glu Leu Gly Val Asp
1955 1960 1965
Ser Leu Ser Ala Leu Glu Leu Arg Asn Arg Leu Thr Thr Ala Thr Gly
1970 1975 1980
Val Arg Leu Ala Thr Thr Thr Val Phe Asp His Pro Asp Val Arg Thr
1985 1990 1995 2000
Leu Ala Gly His Leu Ala Ala Glu Leu Gly Gly Gly Ser Gly Arg Glu
2005 2010 2015
Arg Pro Gly Gly Glu Ala Pro Thr Val Ala Pro Thr Asp Glu Pro Ile
2020 2025 2030
Ala Ile Val Gly Met Ala Cys Arg Leu Pro Gly Gly Val Asp Ser Pro
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Glu Gln Leu Trp Glu Leu Ile Val Ser Gly Arg Asp Thr Ala Ser Ala
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Ala Pro Gly Asp Arg Ser Trp Asp Pro Ala Glu Leu Met Val Ser Asp
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Thr Thr Gly Thr Arg Thr Ala Phe Gly Asn Phe Met Pro Gly Ala Gly
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Thr Gly Ala Gly Pro Tyr Thr Ala Pro Gly Gly Thr Ile Leu Val Thr
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Leu Val Gln Glu Leu Thr Ala Ala Gly Asp Val Val Arg Gly Val Val
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His Ala Ala Gly Leu Pro Gln Gln Val Pro Leu Thr Asp Met Asp Pro
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Leu Ala Asp Leu Cys Pro Glu Ala Glu Leu Phe Leu Leu Phe Ser Ser
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Gly Asp Gln Glu Ala Val Ser Phe Leu Arg Glu Arg Gly Val Arg Pro
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Ile Ala Val Val Gly Met Ala Cys Arg Leu Pro Gly Gly Val His Leu
35 40 45
Pro Gln His Leu Trp Asp Leu Leu Arg Gln Gly His Glu Thr Val Ser
50 55 60
Thr Phe Pro Thr Gly Arg Gly Trp Asp Leu Ala Gly Leu Phe His Pro
65 70 75 80
Asp Pro Asp His Pro Gly Thr Ser Tyr Val Asp Arg Gly Gly Phe Leu
85 90 95
Asp Asp Val Ala Gly Phe Asp Ala Glu Phe Phe Gly Ile Ser Pro Arg
100 105 110
Glu Ala Thr Ala Met Asp Pro Gln Gln Arg Leu Leu Leu Glu Thr Ser
115 120 125
Trp Glu Leu Val Glu Ser Ala Gly Ile Asp Pro His Ser Leu Arg Gly
130 135 140
Thr Pro Thr Gly Val Phe Leu Gly Val Ala Arg Leu Gly Tyr Gly Glu
145 150 155 160
Asn Gly Thr Glu Ala Gly Asp Ala Glu Gly Tyr Ser Val Thr Gly Val
165 170 175
Ala Pro Ala Val Ala Ser Gly Arg Ile Ser Tyr Ala Leu Gly Leu Glu
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Gly Pro Ser Ile Ser Val Asp Thr Ala Cys Ser Ser Ser Leu Val Ala
195 200 205
Leu His Leu Ala Val Glu Ser Leu Arg Leu Gly Glu Ser Ser Leu Ala
210 215 220
Val Val Gly Gly Ala Ala Val Met Ala Thr Pro Gly Val Phe Val Asp
225 230 235 240
Phe Ser Arg Gln Arg Ala Leu Ala Ala Asp Gly Arg Ser Lys Ala Phe
245 250 255
Gly Ala Ala Ala Asp Gly Phe Gly Phe Ser Glu Gly Val Ser Leu Val
260 265 270
Leu Leu Glu Arg Leu Ser Glu Ala Glu Ser Asn Gly His Glu Val Leu
275 280 285
Ala Val Ile Arg Gly Ser Ala Leu Asn Gln Asp Gly Ala Ser Asn Gly
290 295 300
Leu Ala Ala Pro Asn Gly Thr Ala Gln Arg Lys Val Ile Arg Gln Ala
305 310 315 320
Leu Arg Asn Cys Gly Leu Thr Pro Ala Asp Val Asp Ala Val Glu Ala
325 330 335
His Gly Thr Gly Thr Thr Leu Gly Asp Pro Ile Glu Ala Asn Ala Leu
340 345 350
Leu Asp Thr Tyr Gly Arg Asp Arg Asp Pro Asp His Pro Leu Trp Leu
355 360 365
Gly Ser Val Lys Ser Asn Ile Gly His Thr Gln Ala Ala Ala Gly Val
370 375 380
Thr Gly Leu Leu Lys Met Val Leu Ala Leu Arg His Glu Glu Leu Pro
385 390 395 400
Ala Thr Leu His Val Asp Glu Pro Thr Pro His Val Asp Trp Ser Ser

405 410 415
Gly Ala Val Arg Leu Ala Thr Arg Gly Arg Pro Trp Arg Arg Gly Asp
420 425 430
Arg Pro Arg Arg Ala Gly Val Ser Ala Phe Gly Ile Ser Gly Thr Asn
435 440 445
Ala His Val Ile Val Glu Glu Ala Pro Glu Arg Thr Thr Glu Arg Thr
450 455 460
Val Gly Gly Asp Val Gly Pro Val Pro Leu Val Val Ser Ala Arg Ser
465 470 475 480
Ala Ala Ala Leu Arg Ala Gln Ala Ala Gln Val Ala Glu Leu Val Glu
485 490 495
Gly Ser Asp Val Gly Leu Ala Glu Val Gly Arg Ser Leu Ala Val Thr
500 505 510
Arg Ala Arg His Glu His Arg Ala Ala Val Val Ala Ser Thr Arg Ala
515 520 525
Glu Ala Val Arg Gly Leu Arg Glu Val Ala Ala Val Glu Pro Arg Gly
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Glu Asp Thr Val Thr Gly Val Ala Glu Thr Ser Gly Arg Thr Val Val
545 550 555 560
Phe Leu Phe Pro Gly Gln Gly Ser Gln Trp Val Gly Met Gly Ala Glu
565 570 575
Leu Leu Asp Ser Ala Pro Ala Phe Ala Asp Thr Ile Arg Ala Cys Asp
580 585 590
Glu Ala Met Ala Pro Leu Gln Asp Trp Ser Val Ser Asp Val Leu Arg
595 600 605
Gln Glu Pro Gly Ala Pro Gly Leu Asp Arg Val Asp Val Val Gln Pro
610 615 620
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625 630 635 640
Gly Val Thr Pro Ala Ala Val Val Gly His Ser Gln Gly Glu Ile Ala
645 650 655
Ala Ala His Val Ala Gly Ala Leu Ser Leu Ala Asp Ala Ala Arg Leu
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Val Val Gly Arg Ser Arg Leu Leu Arg Ser Leu Ser Gly Gly Gly Gly
675 680 685
Met Ser Ala Val Ala Leu Gly Glu Ala Glu Val Arg Arg Arg Leu Arg
690 695 700
Ser Trp Glu Asp Arg Ile Ser Val Ala Ala Val Asn Gly Pro Arg Ser
705 710 715 720
Val Val Val Ala Gly Glu Pro Glu Ala Leu Arg Glu Trp Gly Arg Glu
725 730 735
Arg Glu Ala Glu Gly Val Arg Val Arg Glu Ile Asp Val Asp Tyr Ala
740 745 750
Ser His Ser Pro Gln Ile Asp Arg Val Arg Asp Glu Leu Leu Thr Val
755 760 765
Thr Gly Glu Ile Glu Pro Arg Ser Ala Glu Ile Thr Phe Tyr Ser Thr
770 775 780
Val Asp Val Arg Ala Val Asp Gly Thr Asp Leu Asp Ala Gly Tyr Trp
785 790 795 800
Tyr Arg Asn Leu Arg Glu Thr Val Arg Phe Ala Asp Ala Met Thr Arg
805 810 815
Leu Ala Asp Ser Gly Tyr Asp Ala Phe Val Glu Val Ser Pro His Pro
820 825 830
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Asp Ala Val Val Val Gly Thr Leu Ser Arg Gly Asp Gly Gly Pro Gly
850 855 860
Ala Phe Leu Arg Ser Ala Ala Thr Ala His Cys Ala Gly Val Asp Val
865 870 875 880
Asp Trp Thr Pro Ala Leu Pro Gly Ala Ala Thr Ile Pro Leu Pro Thr
885 890 895

Tyr Pro Phe Gln Arg Lys Pro Tyr Trp Leu Arg Ser Ser Ala Pro Ala
900 905 910
Pro Ala Ser His Asp Leu Ala Tyr Arg Val Ser Trp Thr Pro Ile Thr
915 920 925
Pro Pro Gly Asp Gly Val Leu Asp Gly Asp Trp Leu Val Val His Pro
930 935 940
Gly Gly Ser Thr Gly Trp Val Asp Gly Leu Ala Ala Ala Ile Thr Ala
945 950 955 960
Gly Gly Gly Arg Val Val Ala His Pro Val Asp Ser Val Thr Ser Arg
965 970 975
Thr Gly Leu Ala Glu Ala Leu Ala Arg Arg Asp Gly Thr Phe Arg Gly
980 985 990
Val Leu Ser Trp Val Ala Thr Asp Glu Arg His Val Glu Ala Gly Ala
995 1000 1005
Val Ala Leu Leu Thr Leu Ala Gln Ala Leu Gly Asp Ala Gly Ile Asp
1010 1015 1020
Ala Pro Leu Trp Cys Leu Thr Gln Glu Ala Val Arg Thr Pro Val Asp
1025 1030 1035 1040
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1045 1050 1055
Val Ala Arg Leu Glu Leu Ala Arg Arg Phe Gly Gly Val Leu Asp Leu
1060 1065 1070
Pro Ala Thr Val Asp Ala Ala Gly Thr Arg Leu Val Ala Ala Val Leu
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Arg Met Pro Ala Ser Leu Val Phe Asp His Pro Thr Val Thr Ala Leu
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Ala Arg Tyr Leu Arg Arg Leu Val Val Gly Asp Ser Asp Pro Thr Pro
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Ser Gly Leu Arg Pro Ala Asp Val Asp Met Val Glu Ala His Gly Thr
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Tyr Gly Arg Asp Arg Asp Arg Pro Leu Trp Leu Gly Ser Val Lys Thr
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1825                1830                1835                1840
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Asp Glu Leu Ser Pro His Ile Asp Trp Ala Asp Gly Lys Val Glu Val
            1860                1865                1870
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Gly Gly Pro Leu Pro Phe Val Leu His Gly Arg Ser Val Gln Thr Val
1925 1930 1935
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1955 1960 1965
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1970 1975 1980
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Ala Pro Ala Val Phe Ala Ala Arg Thr Pro Val Leu Val Phe Pro Gly
2005 2010 2015
Gln Gly Ser Gln Trp Val Gly Met Ala Arg Asp Leu Leu Asp Ser Ser
2020 2025 2030
Glu Val Phe Ala Glu Ser Met Gly Arg Cys Ala Glu Ala Leu Ser Pro
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Pro Thr Ala Glu Leu Asp Glu Phe Leu Ala Val Ala Glu Ala Arg Glu
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Glu Ala Arg Trp Ser Ala Tyr Ala Thr Gly Thr Leu Ala Val Gly Val
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Leu His Ala Thr Gly Ala Thr Ala Val Arg Val Val Ala Thr Pro Ala
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Asp Gln Pro Arg Gly Arg Asp Gly Asp Leu His Arg Leu Glu Trp Val
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Arg Leu Ala Thr Pro Asp Pro Thr Pro Ala Ala Val Val His Val Ala
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Ala Asp Gly Leu Asp Asp Leu Leu Arg Ala Gly Gly Pro Ala Pro Gln
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Ala Val Val Val Arg Tyr Arg Pro Asp Gly Asp Asp Pro Thr Ala Glu
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Gly Val Glu Val Ser Pro Gly Asp Asp Val Pro Arg Pro Gly Ala Ala
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Phe Val Leu Val Asp Gly Asp Pro Glu Thr Pro Pro Ala Val Pro Asp
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50 55 60
Arg Asp Gly Gly Asp Ala Ile Ala Glu Ala Pro Thr Asp Arg Gly Trp
65 70 75 80
Pro Pro Ala Pro Arg Pro Arg Leu Gly Gly Leu Leu Ala Glu Pro Gly
85 90 95
Ala Phe Asp Ala Ala Phe Phe Gly Ile Ser Pro Arg Glu Ala Leu Ala
100 105 110
Thr Asp Pro Gln Gln Arg Leu Met Leu Glu Ile Ser Trp Glu Ala Leu
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Glu Arg Ala Gly Phe Asp Pro Ser Ser Leu Arg Gly Ser Ala Gly Gly
130 135 140
Val Phe Thr Gly Val Gly Ala Val Asp Tyr Gly Pro Arg Pro Asp Glu
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Ala Pro Glu Glu Val Leu Gly Tyr Val Gly Ile Gly Thr Ala Ser Ser
165 170 175
Val Ala Ser Gly Arg Val Ala Tyr Thr Leu Gly Leu Glu Gly Pro Ala
180 185 190
Val Thr Val Asp Thr Ala Cys Ser Ser Gly Leu Thr Ala Val His Leu

195 200 205
Ala Met Glu Ser Leu Arg Arg Asp Glu Cys Thr Leu Val Leu Ala Gly
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Gly Val Thr Val Met Ser Ser Pro Gly Ala Phe Thr Glu Phe Arg Ser
225 230 235 240
Gln Gly Gly Leu Ala Glu Asp Gly Arg Cys Lys Pro Phe Ser Arg Ala
245 250 255
Ala Asp Gly Phe Gly Leu Ala Glu Gly Ala Gly Val Leu Val Leu Gln
260 265 270
Arg Leu Ser Val Ala Arg Ala Glu Gly Arg Pro Val Leu Ala Val Leu
275 280 285
Arg Gly Ser Ala Ile Asn Gln Asp Gly Ala Ser Asn Gly Leu Thr Ala
290 295 300
Pro Ser Gly Pro Ala Gln Arg Arg Val Ile Arg Gln Ala Leu Glu Arg
305 310 315 320
Ala Arg Leu Arg Pro Val Asp Val Asp Tyr Val Glu Ala His Gly Thr
325 330 335
Gly Thr Arg Leu Gly Asp Pro Ile Glu Ala His Ala Leu Leu Asp Thr
340 345 350
Tyr Gly Ala Asp Arg Glu Pro Gly Arg Pro Leu Trp Val Gly Ser Val
355 360 365
Lys Ser Asn Ile Gly His Thr Gln Ala Ala Ala Gly Val Ala Gly Val
370 375 380
Met Lys Thr Val Leu Ala Leu Arg His Arg Glu Ile Pro Ala Thr Leu
385 390 395 400
His Phe Asp Glu Pro Ser Pro His Val Asp Trp Asp Arg Gly Ala Val
405 410 415
Ser Val Val Ser Glu Thr Arg Pro Trp Pro Val Gly Glu Arg Pro Arg
420 425 430
Arg Ala Gly Val Ser Ser Phe Gly Ile Ser Gly Thr Asn Ala His Val
435 440 445
Ile Val Glu Glu Ala Pro Ser Pro Gln Ala Ala Asp Leu Asp Pro Thr
450 455 460
Pro Gly Pro Ala Thr Gly Ala Thr Pro Gly Thr Asp Ala Ala Pro Thr
465 470 475 480
Ala Glu Pro Gly Ala Glu Ala Val Ala Leu Val Phe Ser Ala Arg Asp
485 490 495
Glu Arg Ala Leu Arg Ala Gln Ala Ala Arg Leu Ala Asp Arg Leu Thr
500 505 510
Asp Asp Pro Ala Pro Ser Leu Arg Asp Thr Ala Phe Thr Leu Val Thr
515 520 525
Arg Arg Ala Thr Trp Glu His Arg Ala Val Val Val Gly Gly Gly Glu
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Glu Val Leu Ala Gly Leu Arg Ala Val Ala Gly Gly Arg Pro Val Asp
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565 570 575
Phe Pro Gly Gln Gly Ala Gln Trp Gln Gly Met Ala Arg Asp Leu Leu
580 585 590
Arg Gln Ser Pro Thr Phe Ala Glu Ser Ile Asp Ala Cys Glu Arg Ala
595 600 605
Leu Ala Pro His Val Asp Trp Ser Leu Arg Glu Val Leu Asp Gly Glu
610 615 620
Gln Ser Leu Asp Pro Val Asp Val Val Gln Pro Val Leu Phe Ala Val
625 630 635 640
Met Val Ser Leu Ala Arg Leu Trp Gln Ser Tyr Gly Val Thr Pro Gly
645 650 655
Ala Val Val Gly His Ser Gln Gly Glu Ile Ala Ala Ala His Val Ala
660 665 670
Gly Ala Leu Ser Leu Ala Asp Ala Ala Arg Val Val Ala Leu Arg Ser
675 680 685

Arg Val Leu Arg Arg Leu Gly Gly His Gly Gly Met Ala Ser Phe Gly
690 695 700
Leu His Pro Asp Gln Ala Ala Glu Arg Ile Ala Arg Phe Ala Gly Ala
705 710 715 720
Leu Thr Val Ala Ser Val Asn Gly Pro Arg Ser Val Val Leu Ala Gly
725 730 735
Glu Asn Gly Pro Leu Asp Glu Leu Ile Ala Glu Cys Glu Ala Glu Gly
740 745 750
Val Thr Ala Arg Arg Ile Pro Val Asp Tyr Ala Ser His Ser Pro Gln
755 760 765
Val Glu Ser Leu Arg Glu Glu Leu Leu Ala Ala Leu Ala Gly Val Arg
770 775 780
Pro Val Ser Ala Gly Ile Pro Leu Tyr Ser Thr Leu Thr Gly Gln Val
785 790 795 800
Ile Glu Thr Ala Thr Met Asp Ala Asp Tyr Trp Phe Ala Asn Leu Arg
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Glu Pro Val Arg Phe Gln Asp Ala Thr Arg Gln Leu Ala Glu Ala Gly
820 825 830
Phe Asp Ala Phe Val Glu Val Ser Pro His Pro Val Leu Thr Val Gly
835 840 845
Val Glu Ala Thr Leu Glu Ala Val Leu Pro Pro Asp Ala Asp Pro Cys
850 855 860
Val Thr Gly Thr Leu Arg Arg Glu Arg Gly Gly Leu Ala Gln Phe His
865 870 875 880
Thr Ala Leu Ala Glu Ala Tyr Thr Arg Gly Val Glu Val Asp Trp Arg
885 890 895
Thr Ala Val Gly Glu Gly Arg Pro Val Asp Leu Pro Val Tyr Pro Phe
900 905 910
Gln Arg Gln Asn Phe Trp Leu Pro Val Pro Leu Gly Arg Val Pro Asp
915 920 925
Thr Gly Asp Glu Trp Arg Tyr Gln Leu Ala Trp His Pro Val Asp Leu
930 935 940
Gly Arg Ser Ser Leu Ala Gly Arg Val Leu Val Val Thr Gly Ala Ala
945 950 955 960
Val Pro Pro Ala Trp Thr Asp Val Val Arg Asp Gly Leu Glu Gln Arg
965 970 975
Gly Ala Thr Val Val Leu Cys Thr Ala Gln Ser Arg Ala Arg Ile Gly
980 985 990
Ala Ala Leu Asp Ala Val Asp Gly Thr Ala Leu Ser Thr Val Val Ser
995 1000 1005
Leu Leu Ala Leu Ala Glu Gly Gly Ala Val Asp Asp Pro Ser Leu Asp
1010 1015 1020
Thr Leu Ala Leu Val Gln Ala Leu Gly Ala Ala Gly Ile Asp Val Pro
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Leu Trp Leu Val Thr Arg Asp Ala Ala Ala Val Thr Val Gly Asp Asp
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Val Asp Pro Ala Gln Ala Met Val Gly Gly Leu Gly Arg Val Val Gly
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Val Glu Ser Pro Ala Arg Trp Gly Gly Leu Val Asp Leu Arg Glu Ala
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Asp Ala Asp Ser Ala Arg Ser Leu Ala Ala Ile Leu Ala Asp Pro Arg
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Gly Glu Glu Gln Phe Ala Ile Arg Pro Asp Gly Val Thr Val Ala Arg
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Leu Val Pro Ala Pro Ala Arg Ala Ala Gly Thr Arg Trp Thr Pro Arg
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Gly Thr Val Leu Val Thr Gly Gly Thr Gly Gly Ile Gly Ala His Leu
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Ala Arg Trp Leu Ala Gly Ala Gly Ala Glu His Leu Val Leu Leu Asn
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Arg Arg Gly Ala Glu Ala Ala Gly Ala Ala Asp Leu Arg Asp Glu Leu

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1235 1240 1245
Val Arg Gly Thr Ala Asn Leu Ala Glu Leu Cys Pro Glu Leu Asp Ala
1250 1255 1260
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Gln Gly Leu Arg Ala Met Asp Pro Gln Arg Ala Ile Glu Glu Leu Arg
1330 1335 1340
Thr Thr Leu Asp Ala Gly Asp Pro Trp Val Ser Val Val Asp Leu Asp
1345 1350 1355 1360
Arg Glu Arg Phe Val Glu Leu Phe Thr Ala Ala Arg Arg Arg Pro Leu
1365 1370 1375
Phe Asp Glu Leu Gly Gly Val Arg Ala Gly Ala Glu Glu Thr Gly Gln
1380 1385 1390
Glu Ser Asp Leu Ala Arg Arg Leu Ala Ser Met Pro Glu Ala Glu Arg
1395 1400 1405
His Glu His Val Ala Arg Leu Val Arg Ala Glu Val Ala Ala Val Leu
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Gly His Gly Thr Pro Thr Val Ile Glu Arg Asp Val Ala Phe Arg Asp
1425 1430 1435 1440
Leu Gly Phe Asp Ser Met Thr Ala Val Asp Leu Arg Asn Arg Leu Ala
1445 1450 1455
Ala Val Thr Gly Val Arg Val Ala Thr Thr Ile Val Phe Asp His Pro
1460 1465 1470
Thr Val Asp Arg Leu Thr Ala His Tyr Leu Glu Arg Leu Val Gly Glu
1475 1480 1485
Pro Glu Ala Thr Thr Pro Ala Ala Ala Val Val Pro Gln Ala Pro Gly
1490 1495 1500
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Gly Gly Val Arg Thr Pro Asp Gln Leu Trp Asp Phe Ile Val Ala Asp
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Gly Asp Ala Val Thr Glu Met Pro Ser Asp Arg Ser Trp Asp Leu Asp
1540 1545 1550
Ala Leu Phe Asp Pro Asp Pro Glu Arg His Gly Thr Ser Tyr Ser Arg
1555 1560 1565
His Gly Ala Phe Leu Asp Gly Ala Ala Asp Phe Asp Ala Ala Phe Phe
1570 1575 1580
Gly Ile Ser Pro Arg Glu Ala Leu Ala Met Asp Pro Gln Gln Arg Gln
1585 1590 1595 1600
Val Leu Glu Thr Thr Trp Glu Leu Phe Glu Asn Ala Gly Ile Asp Pro
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1620 1625 1630
Gln Gly Tyr Gly Gln Asn Ala Gln Val Pro Lys Glu Ser Glu Gly Tyr
1635 1640 1645
Leu Leu Thr Gly Gly Ser Ser Ala Val Ala Ser Gly Arg Ile Ala Tyr
1650 1655 1660

Val Leu Gly Leu Glu Gly Pro Ala Ile Thr Val Asp Thr Ala Cys Ser
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Ser Ser Leu Val Ala Leu His Val Ala Ala Gly Ser Leu Arg Ser Gly
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Arg Cys Lys Pro Phe Ser Asp Gln Ala Asp Gly Phe Gly Phe Ala Glu
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Gly Val Ala Val Val Leu Leu Gln Arg Leu Ser Val Ala Val Arg Glu
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1780 1785 1790
Val Ile Arg Arg Ala Trp Gly Arg Ala Gly Val Ser Gly Gly Asp Val
1795 1800 1805
Gly Val Val Glu Ala His Gly Thr Gly Thr Arg Leu Gly Asp Pro Val
1810 1815 1820
Glu Leu Gly Ala Leu Leu Gly Thr Tyr Gly Val Gly Arg Gly Gly Val
1825 1830 1835 1840
Gly Pro Val Val Val Gly Ser Val Lys Ala Asn Val Gly His Val Gln
1845 1850 1855
Ala Ala Ala Gly Val Val Gly Val Ile Lys Val Val Leu Gly Leu Gly
1860 1865 1870
Arg Gly Leu Val Gly Pro Met Val Cys Arg Gly Gly Leu Ser Gly Leu
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1890 1895 1900
Trp Pro Val Gly Val Asp Gly Val Arg Arg Gly Gly Val Ser Ala Phe
1905 1910 1915 1920
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1925 1930 1935
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1940 1945 1950
Val Gly Val Ala Gly Gly Val Val Pro Val Val Leu Ser Ala Lys Thr
1955 1960 1965
Glu Thr Ala Leu Thr Glu Leu Ala Arg Arg Leu His Asp Ala Val Asp
1970 1975 1980
Asp Thr Val Ala Leu Pro Ala Val Ala Ala Thr Leu Ala Thr Gly Arg
1985 1990 1995 2000
Ala His Leu Pro Tyr Arg Ala Ala Leu Leu Ala Arg Asp His Asp Glu
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Trp Gly Ala Trp Ala Gly Asp Gly Met Ala Thr Gly Asp Leu Asp Gly
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Arg Pro Leu Ile Asp Glu Leu Val Thr Ser Ala Pro Val Ala Ala Pro
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Thr Ala Ala Ala Ala Pro Val Pro Ala Met Thr Ala Asp Gln Leu Leu
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Gln Phe Thr Arg Ser His Val Ala Ala Ile Leu Gly His Gln Asp Pro
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Asp Ala Val Gly Leu Asp Gln Pro Phe Thr Glu Leu Gly Phe Asp Ser
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Tyr Pro Pro Gly His Gln Glu Ala Val His Ala Trp Leu Gly Glu Leu
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Leu Thr Ala Leu Gly Ala Tyr Asp Arg Leu Thr Gly Arg Trp Arg Pro

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Gly Glu Trp Pro Asp Asp Gly Trp Gln Ser Thr Trp Pro Phe Gly His
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Asp Arg Val Thr Val Pro Gly Asp His Phe Ser Met Val Gln Glu His
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3185                3190                3195                3200
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    50                  55                  60
Asp His Ala Thr Ala Val Arg Val Leu Asp Asp Pro Thr Phe Thr Arg
65                  70                  75                  80
Ala Thr Gly Arg Thr Pro Glu Trp Met Arg Ala Ala Gly Ala Pro Ala
                85                  90                  95
Ser Thr Trp Ala Gln Pro Phe Arg Asp Val His Ala Ala Ser Trp Asp
            100                 105                 110
Ala Glu Leu Pro Asp Pro Gln Glu Val Glu Asp Arg Leu Thr Gly Leu
        115                 120                 125
Leu Pro Ala Pro Gly Thr Arg Leu Asp Leu Val Arg Asp Leu Ala Trp
    130                 135                 140
Pro Met Ala Ser Arg Gly Val Gly Ala Asp Asp Pro Asp Val Leu Arg
145                 150                 155                 160
Ala Ala Trp Asp Ala Arg Val Gly Leu Asp Ala Gln Leu Thr Pro Gln
                165                 170                 175
Pro Leu Ala Val Thr Glu Ala Ala Ile Ala Ala Val Pro Gly Asp Pro
            180                 185                 190
His Arg Arg Ala Leu Phe Thr Ala Val Glu Met Thr Ala Thr Ala Phe
        195                 200                 205
Val Asp Ala Val Leu Ala Val Thr Ala Thr Ala Gly Ala Ala Gln Arg
    210                 215                 220
Leu Ala Asp Asp Pro Asp Val Ala Ala Arg Leu Val Ala Glu Val Leu
225                 230                 235                 240
Arg Leu His Pro Thr Ala His Leu Glu Arg Arg Thr Ala Gly Thr Glu
                245                 250                 255
Thr Val Val Gly Glu His Thr Val Ala Ala Gly Asp Glu Val Val Val
            260                 265                 270
Val Val Ala Ala Ala Asn Arg Asp Ala Gly Val Phe Ala Asp Pro Asp
        275                 280                 285
Arg Leu Asp Pro Asp Arg Ala Asp Ala Asp Arg Ala Leu Ser Ala Gln
    290                 295                 300
Arg Gly His Pro Gly Arg Leu Glu Glu Leu Val Val Val Leu Thr Thr
305                 310                 315                 320
Ala Ala Leu Arg Ser Val Ala Lys Ala Leu Pro Gly Leu Thr Ala Gly
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<210> 17
<211> 422
<212> PRT
<213> Micromonospora megalomicea

<400> 17
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20 25 30
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35 40 45
Thr Ala Val Pro Val Gly Thr Asp Val Asp Leu Val Asp Phe Met Thr
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His Ala Gly Tyr Asp Ile Ile Asp Tyr Val Arg Ser Leu Asp Phe Ser
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Glu Arg Asp Pro Ala Thr Ser Thr Trp Asp His Leu Leu Gly Met Gln
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Thr Val Leu Thr Pro Thr Phe Tyr Ala Leu Met Ser Pro Asp Ser Leu
100 105 110
Val Glu Gly Met Ile Ser Phe Cys Arg Ser Trp Arg Pro Asp Trp Ser
115 120 125
Ser Gly Pro Gln Thr Phe Ala Ala Ser Ile Ala Ala Thr Val Thr Gly
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Val Ala His Ala Arg Leu Leu Trp Gly Pro Asp Ile Thr Val Arg Ala
145 150 155 160
Arg Gln Lys Phe Leu Gly Leu Leu Pro Gly Gln Pro Ala Ala His Arg
165 170 175
Glu Asp Pro Leu Ala Glu Trp Leu Thr Trp Ser Val Glu Arg Phe Gly
180 185 190
Gly Arg Val Pro Gln Asp Val Glu Glu Leu Val Val Gly Gln Trp Thr
195 200 205
Ile Asp Pro Ala Pro Val Gly Met Arg Leu Asp Thr Gly Leu Arg Thr
210 215 220
Val Gly Met Arg Tyr Val Asp Tyr Asn Gly Pro Ser Val Val Pro Asp
225 230 235 240
Trp Leu His Asp Glu Pro Thr Arg Arg Arg Val Cys Leu Thr Leu Gly
245 250 255
Ile Ser Ser Arg Glu Asn Ser Ile Gly Gln Val Ser Val Asp Asp Leu
260 265 270
Leu Gly Ala Leu Gly Asp Val Asp Ala Glu Ile Ile Ala Thr Val Asp
275 280 285
Glu Gln Gln Leu Glu Gly Val Ala His Val Pro Ala Asn Ile Arg Thr
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Leu Thr Ser Asp Gln Leu Arg Glu Ala Val Arg Arg Val Leu Asp Asp
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Pro Ala Phe Thr Ala Gly Ala Ala Arg Met Arg Ala Asp Met Leu Ala
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                405                 410                 415
Glu Arg Thr Ala Val Gly
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<210> 18
<211> 323
<212> PRT
<213> Micromonospora megalomicea

<400> 18
Met Ser Thr Asp Ala Thr His Val Arg Leu Gly Arg Cys Ala Leu Leu
 1               5                  10                  15
Thr Ser Arg Leu Trp Leu Gly Thr Ala Ala Leu Ala Gly Gln Asp Asp
            20                  25                  30
Ala Asp Ala Val Arg Leu Leu Asp His Ala Arg Ser Arg Gly Val Asn
        35                  40                  45
Cys Leu Asp Thr Ala Asp Asp Asp Ser Ala Ser Thr Ser Ala Gln Val
    50                  55                  60
Ala Glu Glu Ser Val Gly Arg Trp Leu Ala Gly Asp Thr Gly Arg Arg
65                  70                  75                  80
Glu Glu Thr Val Leu Ser Val Thr Val Gly Val Pro Pro Gly Gly Gln
                85                  90                  95
Val Gly Gly Gly Gly Leu Ser Ala Arg Gln Ile Ile Ala Ser Cys Glu
            100                 105                 110
Gly Ser Leu Arg Arg Leu Gly Val Asp His Val Asp Val Leu His Leu
        115                 120                 125
Pro Arg Val Asp Arg Val Glu Pro Trp Asp Glu Val Trp Gln Ala Val
    130                 135                 140
Asp Ala Leu Val Ala Ala Gly Lys Val Cys Tyr Val Gly Ser Ser Gly
145                 150                 155                 160
Phe Pro Gly Trp His Ile Val Ala Ala Gln Glu His Ala Val Arg Arg
                165                 170                 175
His Arg Leu Gly Leu Val Ser His Gln Cys Arg Tyr Asp Leu Thr Ser
            180                 185                 190
Arg His Pro Glu Leu Glu Val Leu Pro Ala Ala Gln Ala Tyr Gly Leu
        195                 200                 205
Gly Val Phe Ala Arg Pro Thr Arg Leu Gly Gly Leu Leu Gly Gly Asp
    210                 215                 220
Gly Pro Gly Ala Ala Ala Ala Arg Ala Ser Gly Gln Pro Thr Ala Leu
225                 230                 235                 240
Arg Ser Ala Val Glu Ala Tyr Glu Val Phe Cys Arg Asp Leu Gly Glu
                245                 250                 255
His Pro Ala Glu Val Ala Leu Ala Trp Val Leu Ser Arg Pro Gly Val
            260                 265                 270
Ala Gly Ala Val Val Gly Ala Arg Thr Pro Gly Arg Leu Asp Ser Ala
        275                 280                 285
Leu Arg Ala Cys Gly Val Ala Leu Gly Ala Thr Glu Leu Thr Ala Leu
    290                 295                 300
Asp Gly Ile Phe Pro Gly Val Ala Ala Ala Gly Ala Ala Pro Glu Ala
305                 310                 315                 320
Trp Leu Arg


<210> 19
<211> 247
<212> PRT
<213> Micromonospora megalomicea

<400> 19
Met Asn Thr Trp Leu Arg Arg Phe Gly Ser Ala Asp Gly His Arg Ala
 1               5                  10                  15
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Arg Leu Tyr Cys Phe Pro His Ala Gly Ala Ala Ala Asp Ser Tyr Leu
            20                  25                  30
Asp Leu Ala Arg Ala Leu Ala Pro Glu Val Asp Val Trp Ala Val Gln
        35                  40                  45
Tyr Pro Gly Arg Gln Asp Arg Arg Asp Glu Arg Ala Leu Gly Thr Ala
    50                  55                  60
Gly Glu Ile Ala Asp Glu Val Ala Ala Val Leu Arg Asp Leu Val Gly
65                  70                  75                  80
Glu Val Pro Phe Ala Leu Phe Gly His Ser Met Gly Ala Leu Val Ala
                85                  90                  95
Tyr Glu Thr Ala Arg Arg Leu Glu Ala Arg Pro Gly Val Arg Pro Leu
            100                 105                 110
Arg Leu Phe Val Ser Gly Gln Thr Ala Pro Arg Val His Glu Arg Arg
        115                 120                 125
Thr Asp Leu Pro Asp Glu Asp Gly Leu Val Glu Gln Met Arg Arg Leu
    130                 135                 140
Gly Val Ser Glu Ala Ala Leu Ala Asp Gln Gly Leu Leu Asp Met Ser
145                 150                 155                 160
Leu Pro Val Leu Arg Ala Asp His Arg Val Leu Arg Ser Tyr Ala Trp
                165                 170                 175
Gln Ala Gly Pro Pro Leu Arg Ala Gly Ile Thr Thr Leu Cys Gly Asp
            180                 185                 190
Thr Asp Pro Leu Thr Thr Val Glu Asp Ala Gln Arg Trp Leu Pro Tyr
        195                 200                 205
Ser Val Val Pro Gly Arg Thr Arg Thr Phe Pro Gly Gly His Phe Tyr
    210                 215                 220
Leu Ala Asp His Val Gly Glu Val Ala Glu Ser Val Ala Pro Asp Leu
225                 230                 235                 240
Leu Arg Leu Thr Pro Thr Gly
                245
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<210> 20
<211> 189
<212> PRT
<213> Micromonospora megalomicea

<400> 20

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Ile Arg Val Gln Asp Asp Asp Ala Asp Arg Leu Ser Arg Asp Glu Leu
 1               5                  10                  15
Thr Ser Ile Ala Leu Val Leu Leu Leu Ala Gly Phe Glu Ala Ser Val
            20                  25                  30
Ser Leu Ile Gly Ile Gly Thr Tyr Leu Leu Leu Thr His Pro Asp Gln
        35                  40                  45
Leu Ala Leu Val Arg Lys Asp Pro Ala Leu Leu Pro Gly Ala Val Glu
    50                  55                  60
Glu Ile Leu Arg Tyr Gln Ala Pro Pro Glu Thr Thr Thr Arg Phe Ala
65                  70                  75                  80
Thr Ala Glu Val Glu Ile Gly Gly Val Thr Ile Pro Ala Tyr Ser Thr
                85                  90                  95
Val Leu Ile Ala Asn Gly Ala Ala Asn Arg Asp Pro Gly Gln Phe Pro
            100                 105                 110
Asp Pro Asp Arg Phe Asp Val Thr Arg Asp Ser Arg Gly His Leu Thr
        115                 120                 125
Phe Gly His Gly Ile His Tyr Cys Met Gly Arg Pro Leu Ala Lys Leu
    130                 135                 140
Glu Gly Glu Val Ala Leu Gly Ala Leu Phe Asp Arg Phe Pro Lys Leu
145                 150                 155                 160
Ser Leu Gly Phe Pro Ser Asp Glu Val Val Trp Arg Arg Ser Leu Leu
                165                 170                 175
Leu Arg Gly Ile Asp His Leu Pro Val Arg Pro Asn Gly
            180                 185
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<210> 21
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic nucleotide DNA duplex

<400> 21
taagaattcg gagatctggc ctcagctcta gac 33

<210> 22
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
<223> Complementary oligo

<400> 22
aattgtctag agctgaggcc agatctccga attcttaat 39

<210> 23
<211> 528
<212> DNA
<213> Micromonospora megalomicea

<400> 23
ttgcagcggt tgtcggtggc ggtgcgggag gggcgtcggg tgttgggtgt ggtggtgggt 60
tcggcggtga atcaggatgg ggcgagtaat gggttggcgg cgccgtcggg ggtggcgcag 120
cagcgggtga ttcggcgggc gtggggtcgt gcgggtgtgt cgggtgggga tgtgggtgtg 180
gtggaggcgc atgggacggg gacgcggttg ggggatccgg tggagttggg ggcgttgttg 240
gggacgtatg gggtgggtcg gggtggggtg ggtccggtgg tggtgggttc ggtgaaggcg 300
aatgtgggtc atgtgcaggc ggcggcgggt gtggtgggtg tgatcaaggt ggtgttgggg 360
ttgggtcggg ggttggtggg tccgatggtg tgtcggggtg ggttgtcggg gttggtggat 420
tggtcgtcgg gtgggttggt ggtggcggat ggggtgcggg ggtggccggt gggtgtggat 480
ggggtgcgtc ggggtggggt gtcggcgttt ggggtgtcgg ggacgaat 528

<210> 24
<211> 528
<212> DNA
<213> Micromonospora megalomicea

<400> 24
ctgcagcggt tgtcggtggc ggtgcgggag gggcgtcggg tgttgggtgt ggtggtgggt 60
tcggcggtga atcaggatgg ggcgagtaat gggttggcgg cgccgtcggg ggtggcgcag 120
cagcgggtga ttcggcgggc gtggggtcgt gcgggtgtgt cgggtgggga tgtgggtgtg 180
gtggaggcgc atgggacggg gacgcggttg ggggatccgg tggagttggg ggcgttgttg 240
gggacgtatg gggtgggtcg gggtggggtg ggtccggtgg tggtgggttc ggtgaaggcg 300
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ttgggtcggg ggttggtggg tccgatggtg tgtcggggtg ggttgtcggg gttggtggat 420
tggtcgtcgg gtgggttggt ggtggcggat ggggtgcggg ggtggccggt gggtgtggat 480
ggggtgcgtc ggggtggggt gtcggcgttt ggggtgtcgg ggacgaat 528

<210> 25
<211> 528
<212> DNA
<213> Micromonospora megalomicea

<220>

<221> misc_feature
<222> (1)...(528)
<223> Sequence with codon changes as described in the
      specification at page 99, line 22 thru 101, line 23

<400> 25
ctgcagcgcc tctccgtcgc cgtccgcgag ggccgccgag tcctcggcgt cgtcgtcggc 60
tcggccgtca accaagacgg cgcgtcaaac ggcctcgccg cgccctccgg cgtcgcccag 120
cagcgcgtca tacgccgcgc gtggggacgc gccggagtat cgggcggcga cgtcggagtc 180
gtcgaggccc acggcaccgg cacccgcctc ggggatcccg tcgagctggg cgccctcctg 240
ggcacgtacg gcgtcggccg cggcggcgtc ggcccggtcg tcgtcggcag cgtcaaggcc 300
aacgtcggcc acgtccaggc cgcggccggc gtcgtcgggg tcatcaaggt cgtcctcggc 360
ctcggccgcg ggctggtcgg cccgatggtc tgccgcggcg gcctcagcgg cctcgtcgac 420
tggtcgtccg gcggcctggt cgtcgcggac ggggtccgcg gctggccggt cggcgtcgac 480
ggcgtccgcc ggggcggcgt ctcggcgttc ggcgtcagcg ggacgaat 528

<210> 26
<211> 291
<212> DNA
<213> Micromonospora megalomicea

<400> 26
ggtggagtgt gatgcggtgg tgtcgtcggt ggtggggttt tcggtgttgg gggtgttgga 60
gggtcggtcg ggtgcgccgt cgttggatcg ggtggatgtg gtgcagccgg tgttgttcgt 120
ggtgatggtg tcgttggcgc ggttgtggcg gtggtgtggg gttgtgcctg cggcggtggt 180
gggtcattcg caggggggaga tcgcggcggc ggtggtggcg gggtgttgt cggtgggtga 240
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<210> 27
<211> 291
<212> DNA
<213> Micromonospora megalomicea

<400> 27
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gggtcggtcg ggtgcgccgt cgttggatcg ggtggatgtg gtgcagccgg tgttgttcgt 120
ggtgatggtg tcgttggcgc ggttgtggcg gtggtgtggg gttgtgcctg cggcggtggt 180
gggtcattcg caggggggaga tcgcggcggc ggtggtggcg gggtgttgt cggtgggtga 240
tggtgcgcgg gtggtggcgt tgcgggcgcg ggcgttgcgg gcgttggccg g 291

<210> 28
<211> 291
<212> DNA
<213> Micromonospora megalomicea

<220>
<221> misc_feature
<222> (1)...(291)
<223> Sequence with codon changes as described in the
      specification at page 99, line 22 thru page 101, line 23

<400> 28
cgtggagtgc gatgcggtcg tgtcgagcgt cgtcggcttc agcgtgctgg gcgtcctgga 60
gggccgcagc ggcgccccga gcctggaccg cgtcgacgtg gtccagccgg tcctgttcgt 120
ggtcatggtc agcctggccc gcctgtggcg ctggtgcggc gtggtcccgg ccgccgtggt 180
cggccacagc cagggcgaga tcgccgccgc ggtcgtggcc ggcgtcctga gcgtcggcga 240
cggcgcccgc gtcgtggccc tgcgcgcccg cgccctgcgc gccctggccg g 291

<210> 29
<211> 24
<212> DNA

<213> Artificial Sequence

<220>
<223> PCR primer

<400> 29
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<210> 30
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<212> DNA
<213> Artificial Sequence

<220>
<223> PCR primer

<400> 30
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<210> 31
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<212> DNA
<213> Artificial Sequence

<220>
<223> PCR primer

<400> 31
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<210> 32
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR primer

<400> 32
aacgcctccc aggagatctc cagca 25

<210> 33
<211> 16
<212> DNA
<213> Artificial Sequence

<220>
<223> Oligo

<400> 33
aattcatagc ctaggt 16

<210> 34
<211> 16
<212> DNA
<213> Artificial Sequence

<220>
<223> Oligo

<400> 34

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(74) Agent: FAVORITO, Carolyn, A.; Morrison & Foerster LLP, Suite 500, 3811 Valley Centre Drive, San Diego, CA 92130-2332 (US).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

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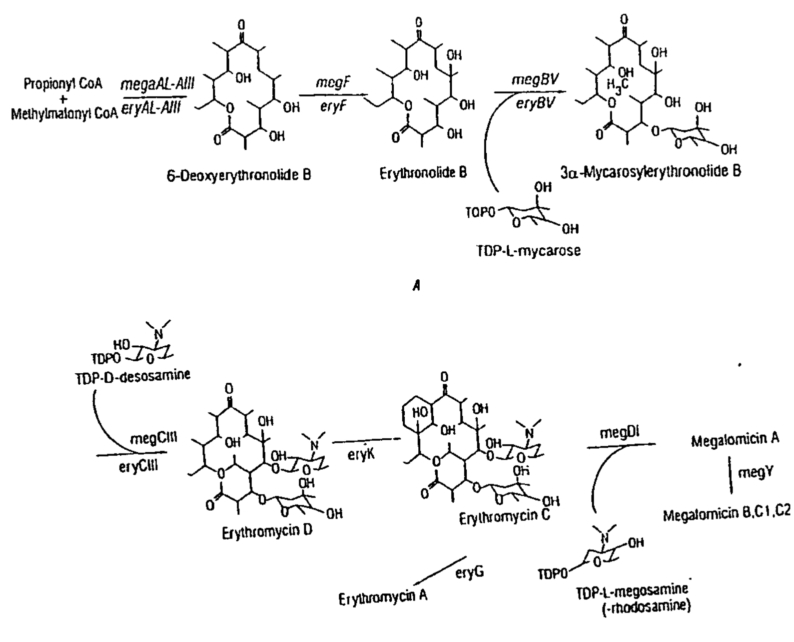
Published:
— *with international search report*

(88) Date of publication of the international search report: 28 February 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: RECOMBINANT MEGALOMICIN BIOSYNTHETIC GENES AND USES THEREOF



WO 01/27284 A3

(57) Abstract: Recombinant nucleic acid, *e.g* DNA compounds that encode all or a portion of the megalomicin polyketide synthase and modification enzymes are used to express recombinant polyketide synthase genes in host cells for the production of megalomicin, megalomicin derivatives, and other polyketides that are useful as antibiotics, motilides, and antiparasitics.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/27433

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/52 C12N15/53 C12N15/54 C12N15/61 C12N15/62
C12N9/04 C12N9/10 C12N9/90 C12P19/62

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EMBL, EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 23630 A (ABBOTT LAB) 3 July 1997 (1997-07-03) the whole document claims 1-22 figures 1-3 ---	1-12,14, 18,19
X	WO 99 05283 A (MENDEZ CARMEN ;SALAS JOSE A (ES); RAYNAL MARIE CECILE (FR); FROMEN) 4 February 1999 (1999-02-04) the whole document claims 1-41 --- -/--	1-12,14, 18,19

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 June 2001	09/07/2001
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer van de Kamp, M

5

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SUMMERS R G ET AL.: "Sequencing and mutagenesis of genes from the erythromycin biosynthetic gene cluster of Saccharopolyspora erythraea that are involved in L-mycarose and D-desosamine production" MICROBIOLOGY, vol. 143, 1 October 1997 (1997-10-01), pages 3251-3262, XP002061260 cited in the application abstract page 3253, right-hand column, line 47 -page 3253, left-hand column, line 19 figures 1-6; table 1 ---	1-12,14, 18,19
X	OLANO C ET AL.: "Analysis of a Streptomyces antibioticus chromosomal region involved in oleandomycin biosynthesis, which encodes two glycosyltransferases responsible for glycosylation of the macrolactone ring" MOLECULAR AND GENERAL GENETICS, vol. 259, no. 3, 1 August 1998 (1998-08-01), pages 299-308, XP002096258 cited in the application abstract page 300, right-hand column, line 46 -page 301, left-hand column, line 17 figures 1,2 ---	1,5-12, 19
X	XUE Y ET AL.: "A gene cluster for macrolide antibiotic biosynthesis in Streptomyces venezuelae: architecture of metabolic diversity" PROC. NATL. ACAD. SCI. USA, vol. 95, October 1998 (1998-10), pages 12111-12116, XP002166926 cited in the application abstract page 12113, left-hand column, line 4-24 figures 1,2; tables 1,2 ---	1,5-12, 19
X	OTTEN S L ET AL.: "Cloning and chracterization of the Streptomyces peucetius dmnZUV genes encoding three enzymes required for biosynthesis of the daunorubicin precursor thymidine diphospho-L-daunosamine" JOURNAL OF BACTERIOLOGY, vol. 179, no. 13, July 1997 (1997-07), pages 4446-4450, XP002166927 abstract figure 1; table 1 ---	1,5-12, 19

-/--

5

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	OTTEN S L ET AL.: "Cloning and characterization of the Streptomyces peucetius dnrQS genes encoding a daunosamine biosynthesis enzyme and a glycosyl transferase involved in daunorubicin biosynthesis" JOURNAL OF BACTERIOLOGY, vol. 177, no. 22, November 1995 (1995-11), pages 6688-6692, XP002166928 abstract figure 1	1,5-12, 19
X	TORKKELL S ET AL.: "Characterization of Streptomyces nogalater genes encoding enzymes involved in glycosylation steps in nogalamycin biosynthesis" MOLECULAR AND GENERAL GENETICS, vol. 256, no. 2, September 1997 (1997-09), pages 203-209, XP002166929 cited in the application abstract figure 1	1,5-12, 19
A	SWAN D G ET AL.: "Characterisation of a Streptomyces antibioticus gene encoding a type I polyketide synthase which has an unusual coding sequence" MOLECULAR AND GENERAL GENETICS, vol. 242, no. 3, 1994, pages 358-362, XP002087278 cited in the application abstract page 358, right-hand column, line 5 -page 361, left-hand column, line 18	1,9
Y	US 3 819 611 A (WEINSTEIN M ET AL) 25 June 1974 (1974-06-25) the whole document	1-12,14, 18-20
Y	MALPARTIDA F ET AL: "Homology between Streptomyces genes coding for synthesis of different polyketides used to clone antibiotic biosynthetic genes" NATURE, vol. 325, 26 February 1987 (1987-02-26), pages 818-821, XP002075972 abstract	1-12,14, 18-20
A	NAKAGAWA A ET AL.: "Structure and stereochemistry of macrolides" MACROLIDE ANTIBIOTICS. OMURA S (ED.). PUBLISHER: ACADEMIC, ORLANDO, FLORIDA, 1984, pages 37-84, XP001006199 page 46, line 25 -page 48, line 4	

-/--

5

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LEONARD KATZ: "Manipulation of modular polyketide synthases" CHEMICAL REVIEWS, vol. 97, no. 7, 1997, pages 2557-2575, XP002103748 the whole document ---	1-12,14, 18,19
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/27433

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E	WO 00 63361 A (KOSAN BIOSCIENCES INC) 26 October 2000 (2000-10-26) page 9, line 3-9 page 14, line 26 -page 16, line 2 claim 3	1-13, 18-20

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(54) Title: RECOMBINANT MEGALOMICIN BIOSYNTHETIC GENES AND USES THEREOF

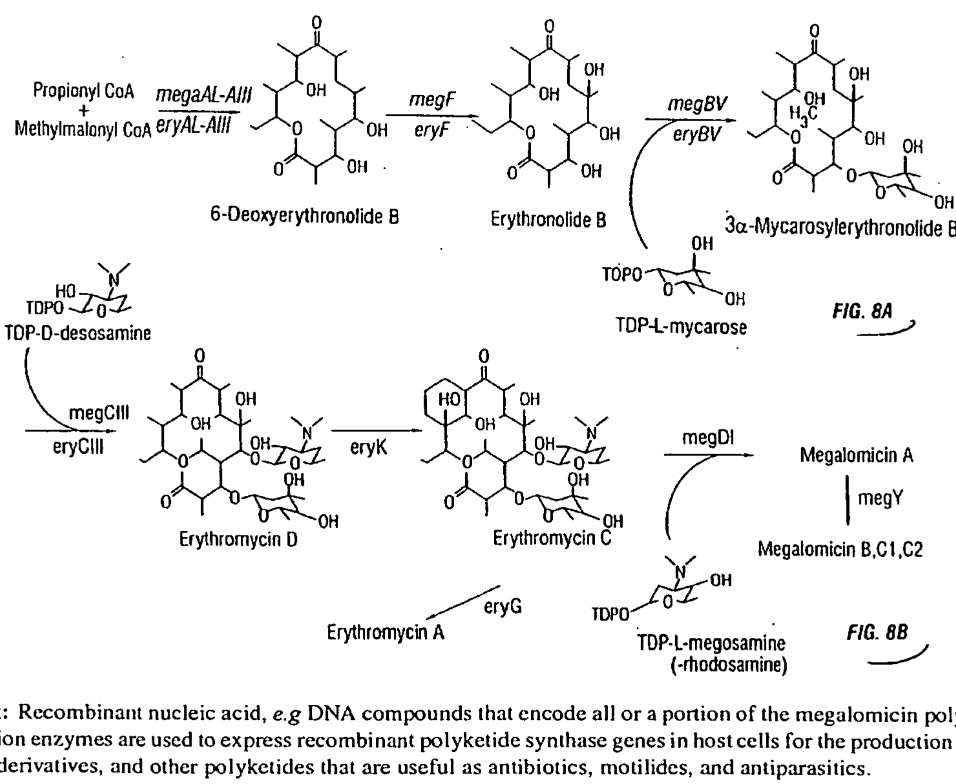

FIG. 8A

FIG. 8B

(57) Abstract: Recombinant nucleic acid, *e.g* DNA compounds that encode all or a portion of the megalomicin polyketide synthase and modification enzymes are used to express recombinant polyketide synthase genes in host cells for the production of megalomicin, megalomicin derivatives, and other polyketides that are useful as antibiotics, motilides, and antiparasitics.

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Title

Recombinant Megalomicin Biosynthetic Genes And Uses Thereof

Cross-Reference to Priority Application

This application claims priority to provisional U.S. patent application Serial No. 60/158,305, filed 8 October 1999, and provisional U.S. patent application Serial No. 60/190,024, filed 17 March 2000 under 35 U.S.C. § 119(e). The content of the above referenced applications is incorporated herein by reference in its entirety.

Field of the Invention

The present invention provides recombinant methods and materials for producing polyketides by recombinant DNA technology. The invention relates to the fields of agriculture, animal husbandry, chemistry, medicinal chemistry, medicine, molecular biology, pharmacology, and veterinary technology.

Background of the Invention

Polyketides represent a large family of diverse compounds synthesized from 2-carbon units through a series of condensations and subsequent modifications. Polyketides occur in many types of organisms, including fungi and mycelial bacteria, in particular, the actinomycetes. There are a wide variety of polyketide structures, and the class of polyketides encompasses numerous compounds with diverse activities. Erythromycin, FK-506, FK-520, megalomicin, narbomycin, oleandomycin, picromycin, rapamycin, spinocyn, and tylosin are examples of such compounds. Given the difficulty in producing polyketide compounds by traditional chemical methodology, and the typically low production of polyketides in wild-type cells, there has been considerable interest in finding improved or alternate means to produce polyketide compounds. See PCT publication Nos. WO 93/13663; WO 95/08548; WO 96/40968; WO 97/02358; and WO 98/27203; United States Patent Nos. 4,874,748; 5,063,155; 5,098,837; 5,149,639; 5,672,491; and 5,712,146; Fu *et al.*, 1994, *Biochemistry 33*: 9321-9326; McDaniel *et al.*, 1993, *Science 262*: 1546-1550; and Rohr, 1995, *Angew.*

Chem. Int. Ed. Engl. 34(8): 881-888, each of which is incorporated herein by reference.

Polyketides are synthesized in nature by polyketide synthase (PKS) enzymes. These enzymes, which are complexes of multiple large proteins, are similar to the synthases that catalyze condensation of 2-carbon units in the biosynthesis of fatty acids. PKS enzymes are encoded by PKS genes that usually consist of three or more open reading frames (ORFs). Two major types of PKS enzymes are known; these differ in their composition and mode of synthesis. These two major types of PKS enzymes are commonly referred to as Type I or "modular" and Type II "iterative" PKS enzymes.

Modular PKSs are responsible for producing a large number of 12-, 14-, and 16-membered macrolide antibiotics including erythromycin, megalomicin, methymycin, narbomycin, oleandomycin, picromycin, and tylosin. Each ORF of a modular PKS can comprise one, two, or more "modules" of ketosynthase activity, each module of which consists of at least two (if a loading module) and more typically three (for the simplest extender module) or more enzymatic activities or "domains." These large multifunctional enzymes (>300,000 kDa) catalyze the biosynthesis of polyketide macrolactones through multistep pathways involving decarboxylative condensations between acyl thioesters followed by cycles of varying ß-carbon processing activities (see O'Hagan, D. *The polyketide metabolites*; E. Horwood: New York, 1991, incorporated herein by reference).

During the past half decade, the study of modular PKS function and specificity has been greatly facilitated by the plasmid-based *Streptomyces coelicolor* expression system developed with the 6-deoxyerythronolide B (6-dEB) synthase (DEBS) genes (see Kao *et al.*, 1994, *Science, 265*: 509-512, McDaniel *et al.*, 1993, *Science 262*: 1546-1557, and U.S. Patent Nos. 5,672,491 and 5,712,146, each of which is incorporated herein by reference). The advantages to this plasmid-based genetic system for DEBS are that it overcomes the tedious and limited techniques for manipulating the natural DEBS host organism, *Saccharopolyspora erythraea*, allows more facile construction of recombinant PKSs, and reduces the complexity of PKS analysis by providing a "clean" host background. This system also expedited construction of the first combinatorial

modular polyketide library in *Streptomyces* (see PCT publication No. WO 98/49315, incorporated herein by reference).

The ability to control aspects of polyketide biosynthesis, such as monomer selection and degree of ß-carbon processing, by genetic manipulation of PKSs has stimulated great interest in the combinatorial engineering of novel antibiotics (see Hutchinson, 1998, *Curr. Opin. Microbiol. 1*: 319-329; Carreras and Santi, 1998, *Curr. Opin. Biotech. 9*: 403-411; and U.S. Patent Nos. 5,712,146 and 5,672,491, each of which is incorporated herein by reference). This interest has resulted in the cloning, analysis, and manipulation by recombinant DNA technology of genes that encode PKS enzymes. The resulting technology allows one to manipulate a known PKS gene cluster either to produce the polyketide synthesized by that PKS at higher levels than occur in nature or in hosts that otherwise do not produce the polyketide. The technology also allows one to produce molecules that are structurally related to, but distinct from, the polyketides produced from known PKS gene clusters.

Megalomicin is a macrolide antibiotic produced by *Micromonospora megalomicea*, a member of the Actinomycetales family of soil bacteria that produces many types of biologically active compounds. Megalomicin is a glycoside of erythromycin A, a widely used antibacterial drug with little or no antimalarial activity. Megalomicin has antibacterial properties similar to those of erythromycin, and in 1998, it was discovered also to have potent antiparasitic activity and low toxicity. The antiparasitic activity may be related to the effect megalomicin has on protein trafficking in eukaryotes, where it appears to inhibit vesicular transport between the medial and trans-Golgi, resulting in under-sialylation of proteins. Hence, megalomicin offers an exciting opportunity to develop a new class of antiparasitic drugs with a different mechanism of action than the drugs currently in use and, therefore, possibly active against drug-resistant forms of *Plasmodium falciparum*.

The number and diversity of megalomicin derivatives have been limited due to the inability to manipulate the PKS genes, which have not previously been available in recombinant form. Genetic systems that allow rapid engineering of the megalomicin biosynthetic genes would be valuable for creating novel compounds for pharmaceutical, agricultural, and veterinary applications. The production of

such compounds could be more readily accomplished if the heterologous expression of the megalomicin biosynthetic genes in *Streptomyces coelicolor* and *S. lividans* and other host cells were possible. The present invention meets these and other needs.

Summary of the Invention

The present invention provides recombinant methods and materials for expressing PKS enzymes and polyketide modification enzymes derived in whole and in part from the megalomicin biosynthetic genes in recombinant host cells. The invention also provides the polyketides produced by such PKS enzymes. The invention provides in recombinant form all of the genes for the proteins that constitute the complete PKS that ultimately results, in *Micromonospora megalomicea,* in the production of megalomicin. Thus, in one embodiment, the invention is directed to recombinant materials comprising nucleic acids with nucleotide sequences encoding at least one domain, module, or protein encoded by a megalomicin PKS gene. In one preferred embodiment of the invention, the DNA compounds of the invention comprise a coding sequence for at least one and preferably two or more of the domains of the loading module and extender modules 1 through 6, inclusive, of the megalomicin PKS.

In one embodiment, the invention provides a recombinant expression vector that comprises a heterologous promoter positioned to drive expression of one or more of the megalomicin biosynthetic genes. In a preferred embodiment, the promoter is derived from another PKS gene. In a related embodiment, the invention provides recombinant host cells comprising one or more expression vectors that produce(s) megalomicin or a megalomicin derivative or precursor. In a preferred embodiment, the host cell is *Streptomyces lividans* or *S. coelicolor*.

In another embodiment, the invention provides a recombinant expression vector that comprises a promoter positioned to drive expression of a hybrid PKS comprising all or part of the megalomicin PKS and at least a part of a second PKS. In a related embodiment, the invention provides recombinant host cells comprising the vector that produces the hybrid PKS and its corresponding polyketide. In a preferred embodiment, the host cell is *Streptomyces lividans* or *S. coelicolor*.

In a related embodiment, the invention provides recombinant materials for the production of libraries of polyketides wherein the polyketide members of the library are synthesized by hybrid PKS enzymes of the invention. The resulting polyketides can be further modified to convert them to other useful compounds, such as antibiotics, motilides, and antiparasitics, typically through hydroxylation and/or glycosylation. Modified macrolides provided by the invention that are useful intermediates in the preparation of antiparasitics are of particular benefit.

In another related embodiment, the invention provides a method to prepare a nucleic acid that encodes a modified PKS, which method comprises using the megalomicin PKS encoding sequence as a scaffold and modifying the portions of the nucleotide sequence that encode enzymatic activities, either by mutagenesis, inactivation, deletion, insertion, or replacement. The thus modified megalomicin PKS encoding nucleotide sequence can then be expressed in a suitable host cell and the cell employed to produce a polyketide different from that produced by the megalomicin PKS. In addition, portions of the megalomicin PKS coding sequence can be inserted into other PKS coding sequences to modify the products thereof.

In another related embodiment, the invention is directed to a multiplicity of cell colonies, constituting a library of colonies, wherein each colony of the library contains an expression vector for the production of a modular PKS derived in whole or in part from the megalomicin PKS. Thus, at least a portion of the modular PKS is identical to that found in the PKS that produces megalomicin and is identifiable as such. The derived portion can be prepared synthetically or directly from DNA derived from organisms that produce megalomicin. In addition, the invention provides methods to screen the resulting polyketide and antibiotic libraries.

The invention also provides novel polyketides, motilides, antibiotics, antiparasitics and other useful compounds derived therefrom. The compounds of the invention can also be used in the manufacture of another compound. In a preferred embodiment, the compounds of the invention are formulated in a mixture or solution for administration to an animal or human.

In a specific embodiment, the invention provides an isolated nucleic acid fragment comprising a nucleotide sequence encoding a domain of megalomicin polyketide synthase (PKS) or a megalomicin modification enzyme. The isolated

nucleic acid fragment can be a DNA or a RNA. Preferably, the isolated nucleic acid fragment is a recombinant DNA compound.

The isolated nucleic acid fragment can comprise a single, multiple or all the open reading frame(s) (ORF) of the megalomicin PKS or a megalomicin modification enzyme. Exemplary ORFs of megalomicin PKS include the ORFs of the *megAI*, *megAII* and *megAIII* genes. The isolated nucleic acid fragment can also encode a single, multiple, or all of the domains of the megalomicin PKS. Exemplary domains of the megalomicin PKS include a TE domain, a KS domain, an AT domain, an ACP domain, a KR domain, a DH domain and an ER domain. In a preferred embodiment, the nucleic acid fragment encodes a module of the megalomicin PKS. In another preferred embodiment, the nucleic acid fragment encodes the loading module, a thioesterase domain, and all six extender modules of the megalomicin PKS.

Megalomicin modification enzymes include those enzymes involved in the conversion of 6-dEB into a megalomicin such as the enzymes encoded by the *megF*, *meg BV*, *megCIII*, *megK*, *megDI* and *megG* (renamed *megY*) genes. Megalomicin modification enzymes also include those enzymes involved in the biosynthesis of mycarose, megosamine or desosamine, which are used as biosynthetic intermediates in the biosynthesis of various megalomicin species and other related polyketides. The enzymes that are involved in biosynthesis of mycarose, megosamine or desosamine are described in Figures 5 and 10.

In a preferred embodiment, the invention provides an isolated nucleic acid fragment which hybridizes to a nucleic acid having a nucleotide sequence set forth in the SEQ. ID NO:1, under low, medium or high stringency. More preferably, the nucleic acid fragment comprises, consists or consists essentially of a nucleic acid having a nucleotide sequence set forth in the SEQ. ID NO:1.

In another specific embodiment, the invention provides a substantially purified polypeptide, which is encoded by a nucleic acid fragment comprising a nucleotide sequence encoding a domain of megalomicin polyketide synthase (PKS) or a megalomicin modification enzyme. The polypeptide can comprise a single domain, multiple domains or a full-length megalomicin PKS or megalomicin modification enzyme. Functional fragments, analogs or derivatives of the megalomicin PKS or megalomicin modification enzyme polypeptides are

also provided. Preferably, such fragments, analogs or derivatives can be recognized by an antibody raised against a megalomicin PKS or megalomicin modification enzyme. Also preferably, such fragments, analogs or derivatives comprise an amino acid sequence that has at least 60% identity, more preferably at least 90% identity, to their wild type counterparts.

In still another specific embodiment, the invention provides an antibody, or a fragment or derivative thereof, which immuno-specifically binds to a domain of megalomicin polyketide synthase (PKS) or a megalomicin modification enzyme. The antibody can be a monoclonal or polyclonal antibody or an antibody fragment. Preferably, the antibody is a monoclonal antibody.

In yet another specific embodiment, the invention provides a recombinant DNA expression vector comprising the recombinant DNA compound encoding at least a domain of the megalomicin PKS or a megalomicin modification enzyme, wherein said domain is operably linked to a promoter. Preferably, the recombinant DNA expression vector further comprises an origin of replication or a segment of DNA that enables chromosomal integration.

In yet another specific embodiment, the invention provides a recombinant host cell comprising the above-described recombinant DNA expression vector encoding at least a domain of megalomicin PKS or the megalomicin modification enzyme. The recombinant host cells can be any suitable host cells including animal, mammalian, plant, fungal, yeast, and bacterial cells. Preferably, the recombinant host cells are *Streptomyces* cells, such as *Streptomyces lividans* and *S. coelicolor* cells, or *ccharopolyspora* cells, such as *Saccharopolyspora erythraea* cells. Also preferably, the recombinant host cells do not produce megalomicin in their untransformed, non-recombinant state.

When the recombinant host cell contains nucleic acid encoding more than one megalomicin PKS or megalomicin modification enzyme, or domains thereof, such nucleic acid material can be located at a single genetic locus, *e.g.*, on a single plasmid or at a single chromosomal locus, or at different genetic loci, *e.g.*, on separate plasmids and/or chromosomal loci. In one example, the invention provides a recombinant host cell, which comprises at least two separate autonomously replicating recombinant DNA expression vectors, and each of said vectors comprises a recombinant DNA compound encoding a megalomicin PKS

domain or a megalomicin modification enzyme operably linked to a promoter. In another example, the invention provides a recombinant host cell, which comprises at least one autonomously replicating recombinant DNA expression vector and at least one modified chromosome, each of said vector(s) and each of said modified chromosome comprises a recombinant DNA compound encoding a megalomicin PKS domain or a megalomicin modification enzyme operably linked to a promoter. Preferably, the autonomously replicating recombinant DNA expression vector and/or the modified chromosome further comprises distinct selectable markers.

In a preferred embodiment, the cell comprises three different vectors, one of which is integrated into the chromosome and two of which are autonomously replicating, and each of the vectors comprises a *meg* PKS gene. Optionally, one or more of the *meg* PKS genes contains one or more domain alterations, such as a deletion or substitution of a meg PKS domain with a domain from another PKS.

In yet another specific embodiment, the invention provides a hybrid PKS, which is produced from a recombinant gene that comprises at least a portion of a megalomicin PKS gene and at least a portion of a second PKS gene for a polyketide other than megalomicin. For example, and without limitation, the second PKS gene can be a narbonolide PKS gene, an oleandolide PKS gene, or a rapamycin PKS gene. In one embodiment, the hybrid PKS is composed of a loading module and six extender modules, wherein at least one domain of any one of extender modules 1 through 6, inclusive, is a domain of an extender module of megalomicin PKS. In another preferred embodiment, the hybrid PKS comprises a megalomicin PKS that has a non-functional KS domain in module 1.

In yet another specific embodiment, the invention provides a method of producing a polyketide, which method comprises growing the recombinant host cell comprising a recombinant DNA expression vector encoding at least a domain of the megalomicin PKS or a megalomicin modification enzyme under conditions whereby the megalomicin PKS domain or the megalomicin modification enzyme comprised by the recombinant expression vector is produced and the polyketide is synthesized by the cell, and recovering the synthesized polyketide. Preferably, the recombinant host cell comprises a recombinant expression vector that encodes at least a portion of a *megAI*, *megAII*, or *megAIII* gene.

These and other embodiments of the invention are described in more detail in the following description, the examples, and claims set forth below.

Brief Description of the Figures

Figure 1 shows restriction site and function maps of the insert DNA in cosmids pKOS079-138B, pKOS079-93D, pKOS079-93A, and pKOS079-124B of the invention. Various restriction sites (*Xho*I, *Bgl*II, *Nsi*I) are also shown. The location of the megalomicin biosynthetic genes is shown below the solid lines indicating the cosmid inserts. The genes are shown as arrows pointing in the direction of transcription. The approximate size (in kilobase (kb) pairs) of the gene cluster is indicated in 5000 bp (i.e., 5K, 10K, and the like.) increments on a solid bar beneath the arrows indicating the genes.

Figure 2 shows a more detailed map of the megalomicin biosynthetic gene cluster. The various open reading frames are shown as arrows pointing in the direction of transcription. A line indicates the size in base pairs (in 1000 bp increments) of the gene cluster. The various domains of the megalomicin PKS are also shown. Other genes of the megalomicin biosynthetic gene cluster not shown in this Figure are located in the insert DNA of cosmids pKOS0138B and pKOS0124B.

Figure 3 shows the structures of the megalomicins, azithromycin and erythromycin A.

Figure 4 shows the modules and domains of DEBS and the megalomicin PKS.

Figure 5 shows the compounds and reactions in the erythromycin biosynthetic pathway and also for megalomicin biosynthesis. Genes that produce the various enzymes that catalyze each of the steps in the biosynthetic pathway are indicated.

Figure 6 shows the biosynthetic pathway for the formation of desosamine, rhodosamine, and mycarose, as well as the genes that produce the various enzymes that catalyze each of the steps in the biosynthetic pathway.

Figure 7 depicts nucleotide and amino acid sequence of *Micromonospora megalomicea* megalomicin biosynthetic genes (GenBank Accession No. AF263245, incorporated herein by reference).

Figure 8 depicts the biosynthesis of the erythromycins and megalomicins and the enzymes that mediate the biosynthesis of each.

Figure 9 depicts the cloned megalomicin biosynthetic gene cluster and certain cosmids of the invention that comprise portions of the cluster.

Figure 10 depicts the biosynthesis of megosamine, mycarose, and desosamine.

Detailed Description of the Invention

The present invention provides useful compounds and methods for producing polyketides in recombinant host cells. As used herein, the term recombinant refers to a compound or composition produced by human intervention. The invention provides recombinant DNA compounds encoding all or a portion of the megalomicin biosynthetic genes. The invention provides recombinant expression vectors useful in producing the megalomicin PKS and hybrid PKSs composed of a portion of the megalomicin PKS in recombinant host cells. The invention also provides the polyketides produced by the recombinant PKS and polyketide modification enzymes.

To appreciate the many and diverse benefits and applications of the invention, the description of the invention below is organized as follows. In Section I, common definitions used throughout this application are provided. In Section II, structural and functional characteristics of megalomicin are described. In Section III, the recombinant megalomicin biosynthetic genes and other recombinant nucleic acids provided by the invention are described. In Section IV, polypeptides and proteins encoded by the megalomicin biosynthetic genes and antibodies that specifically bind to such polypeptides and proteins provided by the invention are described. In Section V, methods for heterologous expression of the megalomicin biosynthetic genes provided by the invention are described. In Section VI, the hybrid PKS genes provided by the invention are described. In Section VII, host cells containing multiple megalomicin biosynthetic genes and nucleic acid fragments on separate express vectors provided by the invention are described. In Section VIII, the polyketide compounds provided by the invention and pharmaceutical compositions of those compounds are described. The detailed description is followed by working examples illustrating the invention.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art to which this invention belongs. All patents, applications, published applications and other publications and sequences from GenBank and other data bases referred to herein are incorporated by reference in their entirety.

Section I. Definitions

As used herein, domain refers to a portion of a molecule, *e.g.*, proteins or nucleic acids, that is structurally and/or functionally distinct from another portion of the molecule.

As used herein, antibody includes antibody fragments, such as Fab fragments, which are composed of a light chain and the variable region of a heavy chain.

As used herein, biological activity refers to the *in vivo* activities of a compound or physiological responses that result upon *in vivo* administration of a compound, composition or other mixture. Biological activity, thus, encompasses therapeutic effects and pharmaceutical activity of such compounds, compositions and mixtures. Biological activities may be observed in in vitro systems designed to test or use such activities.

As used herein, a combination refers to any association between two or among more items.

As used herein, a composition refers to any mixture. It may be a solution, a suspension, liquid, powder, a paste, aqueous, non-aqueous or any combination thereof.

As used herein, derivative or analog of a molecule refers to a portion derived from or a modified version of the molecule.

As used herein, operably linked, operatively linked or operationally associated refers to the functional relationship of DNA with regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences. For example, operative linkage of DNA to a promoter refers to the physical and functional relationship between the DNA and the promoter such that the transcription of such DNA is initiated from the promoter by an RNA polymerase that specifically recognizes,

binds to and transcribes the DNA. To optimize expression and/or *in vitro* transcription, it may be helpful to remove, add or alter 5' untranslated portions of the clones to eliminate extra, potentially inappropriate alternative translation initiation (*i.e.*, start) codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites (see, *e.g.*, Kozak, *J. Biol. Chem.*, *266*:19867-19870 (1991)) can be inserted immediately 5' of the start codon and may enhance expression. The desirability of (or need for) such modification may be empirically determined.

As used herein, pharmaceutically acceptable salts, esters or other derivatives of the conjugates include any salts, esters or derivatives that may be readily prepared by those of skill in this art using known methods for such derivatization and that produce compounds that may be administered to animals or humans without substantial toxic effects and that either are pharmaceutically active or are prodrugs.

As used herein, a promoter region or promoter element refers to a segment of DNA or RNA that controls transcription of the DNA or RNA to which it is operatively linked. The promoter region includes specific sequences that are sufficient for RNA polymerase recognition, binding and transcription initiation. This portion of the promoter region is referred to as the promoter. In addition, the promoter region includes sequences that modulate this recognition, binding and transcription initiation activity of RNA polymerase. These sequences may be *cis* acting or may be responsive to *trans* acting factors. Promoters, depending upon the nature of the regulation, may be constitutive or regulated.

As used herein: stringency of hybridization in determining percentage mismatch is as follows: (1) high stringency: 0.1 x SSPE, 0.1% SDS, 65°C; (2) medium stringency: 0.2 x SSPE, 0.1% SDS, 50°C; and (3) low stringency: 1.0 x SSPE, 0.1% SDS, 50°C. Equivalent stringencies may be achieved using alternative buffers, salts and temperatures.

The term substantially identical or homologous or similar varies with the context as understood by those skilled in the relevant art and generally means at least 70%, preferably means at least 80%, more preferably at least 90%, and most preferably at least 95% identity.

As used herein, substantially identical to a product means sufficiently similar so that the property of interest is sufficiently unchanged so that the substantially identical product can be used in place of the product.

As used herein, isolated means that a substance is either present in a preparation at a concentration higher than that substance is found in nature or in its naturally occurring state or that the substance is present in a preparation that contains other materials with which the substance is not associated with in nature. As an example of the latter, an isolated meg PKS protein includes a meg PKS protein expressed in a *Streptomyces coelicolor* or *S. lividans* host cell.

As used herein, substantially pure means sufficiently homogeneous to appear free of readily detectable impurities as determined by standard methods of analysis, such as thin layer chromatography (TLC), gel electrophoresis and high performance liquid chromatography (HPLC), used by those of skill in the art to assess such purity, or sufficiently pure such that further purification would not detectably alter the physical and chemical properties, such as enzymatic and biological activities, of the substance. Methods for purification of the compounds to produce substantially chemically pure compounds are known to those of skill in the art. A substantially chemically pure compound may, however, be a mixture of stereoisomers or isomers. In such instances, further purification might increase the specific activity of the compound.

As used herein, vector or plasmid refers to discrete elements that are used to introduce heterologous DNA into cells for either expression or replication thereof. Selection and use of such vehicles are well known within the skill of the artisan. An expression vector includes vectors capable of expressing DNAs that are operatively linked with regulatory sequences, such as promoter regions, that are capable of effecting expression of such DNA fragments. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome.

Section II. Megalomicins

The megalomicins were discovered in 1969 at Schering Corp. as antibacterial agents produced by *Micromonospora megalomicea* (see Weinstein *et al.*, 1969, *J. Antibiotics 22*: 253-258, and U.S. Patent No. 3,632,750, both of which are incorporated herein by reference). Although the initial structural assignment was in error, a thorough reassessment of NMR data coupled with an X-ray crystal structure of a megalomicin A derivative (see Nakagawa and Omura, "Structure and Stereochemistry of Macrolides" in *Macrolide Antibiotics* (S. Omura, ed.), Academic Press, NY, 1984, incorporated herein by reference) established the structures shown in Figure 3. The megalomicins are 6-*O*-glycosides of erythromycin C with acetyl or propionyl groups esterified at the 3''' or 4''' hydroxyls of the mycarose sugar at the C-3-position. The C-6 sugar has been named "megosamine," although it had been identified 5 to 10 years earlier as L-rhodosamine or *N*-dimethyldaunosamine, deoxyamino sugars commonly present in the anthracycline antitumor drugs. The antibacterial potency, spectrum of activity, and toxicity (LD_{50} acute, 7-7.5 g/kg s.c. or oral; subacute, >500 mg/kg) of the megalomicins is similar to that of erythromycin A.

The megalomicins have two modes of biological activity. As antibacterials, they act like the erythromycins, which inhibit protein synthesis at the translocation step by selective binding to the bacterial 50*S* ribosomal RNA. They also affect

protein trafficking in eukaryotic cells (see Bonay *et al.*, 1996, *J. Biol. Chem.* *271*:3719-3726, incorporated herein by reference). Although the mechanism of action is not entirely clear, it appears to involve inhibition of vesicular transport between the medial and trans Golgi, resulting in under-sialylation of proteins. The megalomicins also strongly inhibit the ATP-dependent acidification of lysosomes *in vivo* (see Bonay *et al.*, 1997, *J. Cell. Sci.* *110*:1839-1849, incorporated herein by reference) and cause an anomalous glycosylation of viral proteins, which may be responsible for their antiviral activity against herpes (Tox_{50}, 70-100 μM; see Alarcon *et al.*, 1984, *Antivir. Res.* 4:231-243, and Alarcon *et al.*, 1988, *FEBS Lett.* *231*:207-211, both of which are incorporated herein by reference).

Strikingly, the megalomicins are potent antiparasitic agents, showing an IC_{50} of 1 μg/ml in blocking intracellular replication of *Plasmodium falciparum* infected erythrocytes (see Bonay *et al.*, 1998, *Antimicrob. Agents Chemother.* *42*:2668-2673, incorporated herein by reference). The megalomicins are effective against *Trypanosoma cruzi* and *T. brucei* (IC_{50}, 0.2-2 μg/ml) plus *Leishmania donovani* and *L. major* promastigotes (IC_{50}, 3 and 8 μg/ml, respectively). Megalomicin is also active against the intracellular replicative, amastigote form of *T. cruzi*, completely preventing its replication in infected murine LLC/MK2 macrophages at a dose of 5 μg/ml. Importantly, the effective drug concentration is 500-fold less than the acute LD_{50} in mammals, and there is no toxicity to BALB/c mice at doses (50 mg/kg) that are completely curative for *T. brucei* infections. Because the erythromycins do not have such activity, although azithromycin (Figure 3) has been reported to be an effective acute and prophylactic treatment for malaria caused by *P. vivax* and *P. falciparum* (see Taylor *et al.*, 1999, *Clin. Infect. Dis.* 28:74-81, incorporated herein by reference), the antiparasitic action of the megalomicins is unique and probably related to the presence of the deoxyamino sugar megosamine at C-6 (Figure 3). Consequently, the megalomicins could be developed into potent antimalarial drugs with a high therapeutic index and be active against *P. falciparum* and other species that are resistant to currently used classes of antimalarials. They also could lead to potent antiparasitic agents against leishmaniasis, trypanosomiasis, and Chagas' disease. In view of the widespread use of the erythromycins and their good oral availability plus the low mammalian toxicity of macrolides in general, the megalomicins could be used prophylactically

to combat malaria, and as fermentation products, the megalomicins should be relatively inexpensive to produce.

The megalomicins belong to the polyketide class of natural products whose members have diverse structural and pharmacological properties (see Monaghan and Tkacz, 1990, *Annu. Rev. Microbiol. 44*: 271, incorporated herein by reference). The megalomicins are assembled by polyketide synthases through successive condensations of activated coenzyme-A thioester monomers derived from small organic acids such as acetate, propionate, and butyrate. Active sites required for condensation include an acyltransferase (AT), acyl carrier protein (ACP), and beta-ketoacylsynthase (KS). Each condensation cycle results in a ß-keto group that undergoes all, some, or none of a series of processing activities. Active sites that perform these reactions include a ketoreductase (KR), dehydratase (DH), and enoylreductase (ER). Thus, the absence of any beta-keto processing domain results in the presence of a ketone, a KR alone gives rise to a hydroxyl, a KR and DH result in an alkene, while a KR, DH, and ER combination leads to complete reduction to an alkane. After assembly of the polyketide chain, the molecule typically undergoes cyclization(s) and post-PKS modification (e.g. glycosylation, oxidation, acylation) to achieve the final active compound.

Macrolides such as erythromycin and megalomicin are synthesized by modular PKSs (see Cane *et al.*, 1998, *Science 282*: 63, incorporated herein by reference). For illustrative purposes, the PKS that produces the erythromycin polyketide (6-deoxyerythronolide B synthase or DEBS; see U.S. Patent No. 5,824,513, incorporated herein by reference) is shown in Figure 4. DEBS is the most characterized and extensively used modular PKS system. DEBS is particularly relevant to the present invention in that it synthesizes the same polyketide, 6-deoxyerythronolide B (6-dEB), synthesized by the megalomicin PKS. In modular PKS enzymes such as DEBS and the megalomicin PKS, the enzymatic steps for each round of condensation and reduction are encoded within a single "module" of the polypeptide (i.e., one distinct module for every condensation cycle). DEBS consists of a loading module and 6 extender modules and a chain terminating thioesterase (TE) domain within three extremely large polypeptides encoded by three open reading frames (ORFs, designated *eryAI*, *eryAII*, and *eryAIII*).

Each of the three polypeptide subunits of DEBS (DEBSI, DEBSII, and DEBSIII) contains 2 extender modules, DEBSI additionally contains the loading module. Collectively, these proteins catalyze the condensation and appropriate reduction of 1 propionyl CoA starter unit and 6 methylmalonyl CoA extender units. Modules 1, 2, 5, and 6 contain KR domains; module 4 contains a complete set, KR/DH/ER, of reductive and dehydratase domains; and module 3 contains no functional reductive domain. Following the condensation and appropriate dehydration and reduction reactions, the enzyme bound intermediate is lactonized by the TE at the end of extender module 6 to form 6-dEB.

More particularly, the loading module of DEBS consists of two domains, an acyl-transferase (AT) domain and an acyl carrier protein (ACP) domain. In other PKS enzymes, the loading module is not composed of an AT and an ACP but instead utilizes an inactivated KS, an AT, and an ACP. This inactivated KS is in most instances called KS^Q, where the superscript letter is the abbreviation for the amino acid, glutamine, that is present instead of the active site cysteine required for activity. The AT domain of the loading module recognizes a particular acyl-CoA (propionyl for DEBS, which can also accept acetyl) and transfers it as a thiol ester to the ACP of the loading module. Concurrently, the AT on each of the extender modules recognizes a particular extender-CoA (methylmalonyl for DEBS) and transfers it to the ACP of that module to form a thioester. Once the PKS is primed with acyl- and malonyl-ACPs, the acyl group of the loading module migrates to form a thiol ester (trans-esterification) at the KS of the first extender module; at this stage, extender module 1 possesses an acyl-KS and a methylmalonyl ACP. The acyl group derived from the loading module is then covalently attached to the alpha-carbon of the malonyl group to form a carbon-carbon bond, driven by concomitant decarboxylation, and generating a new acyl-ACP that has a backbone two carbons longer than the loading unit (elongation or extension). The growing polyketide chain is transferred from the ACP to the KS of the next module, and the process continues.

The polyketide chain, growing by two carbons each module, is sequentially passed as a covalently bound thiol ester from module to module, in an assembly line-like process. The carbon chain produced by this process alone would possess a ketone at every other carbon atom, producing a polyketone, from which the

name polyketide arises. Commonly, however, the beta keto group of each two-carbon unit is modified just after it has been added to the growing polyketide chain but before it is transferred to the next module by either a KR, a KR plus a DH, or a KR, a DH, and an ER. As noted above, modules may contain additional enzymatic activities as well.

Once a polyketide chain traverses the final extender module of a PKS, it encounters the releasing domain or thioesterase found at the carboxyl end of most PKSs. Here, the polyketide is cleaved from the enzyme and cyclyzed. The resulting polyketide can be modified further by tailoring or modification enzymes; these enzymes add carbohydrate groups or methyl groups, or make other modifications, i.e., oxidation or reduction, on the polyketide core molecule. For example, the final steps in conversion of 6-dEB to erythromycin A include the actions of a number of modification enzymes, such as: C-6 hydroxylation, attachment of mycarose and desosamine sugars, C-12 hydroxylation (which produces erythromycin C), and conversion of mycarose to cladinose via *O*-methylation, as shown in Figure 5.

With this overview of PKS and post-PKS modification enzymes, one can better appreciate the recombinant megalomicin biosynthetic genes provided by the invention and their function, as described in the following Section.

Section III: The Megalomicin Biosynthetic Genes and Nucleic Acid Fragments

The megalomicin PKS was isolated and cloned by the following procedure. Genomic DNA was isolated from a megalomicin producing strain of *Micromonospora megalomicea* subsp. nigra (ATCC 27598), partially digested with a restriction enzyme, and cloned into a commercially available cosmid vector to produce a genomic library. This library was then probed with probe generated from the erythromycin biosynthetic genes as well as from cosmids identified as containing sequences homologous to erythromycin biosynthetic genes. This probing identified a set of cosmids, which were analyzed by DNA sequence analysis and restriction enzyme digestion, which revealed that the desired DNA had been isolated and that the entire PKS gene cluster was contained in overlapping segments on four of the cosmids identified. Figure 1 shows the cosmids, and the portions of the megalomicin biosynthetic gene cluster in the

insert DNA of the cosmids. Figure 1 shows that the complete megalomicin biosynthetic gene cluster is contained within the insert DNA of cosmids pKOS079-138B, pKOS079-124B, pKOS079-93D, and pKOS079-93A. Each of these cosmids has been deposited with the American Type Culture Collection in accordance with the terms of the Budapest Treaty (cosmid pKOS079-138B is available under accession no. **ATCC** _____; cosmid pKOS079-124B is available under accession no. **ATCC** _____; cosmid pKOS079-93D is available under accession no. **ATCC** _____; and cosmid pKOS079-93A is available under accession no. **ATCC** _____). Various additional reagents of the invention can be isolated from these cosmids. DNA sequence analysis was also performed on the various subclones of the invention, as described herein. Further analysis of these cosmids and subclones prepared from the cosmids facilitated the identification of the location of various megalomicin biosynthetic genes, including the ORFs encoding the PKS, modules encoded by those ORFs, and coding sequences for megalomicin modification enzymes. The location of these genes and modules is shown on Figure 2.

Those of skill in the art will recognize that, due to the degenerate nature of the genetic code, a variety of DNA compounds differing in their nucleotide sequences can be used to encode a given amino acid sequence of the invention. The native DNA sequence encoding the megalomicin PKS and other biosynthetic enzymes and other biosynthetic enzymes of *Micromonospora megalomicea* is shown herein merely to illustrate a preferred embodiment of the invention, and the invention includes DNA compounds of any sequence that encode the amino acid sequences of the polypeptides and proteins of the invention. In similar fashion, a polypeptide can typically tolerate one or more amino acid substitutions, deletions, and insertions in its amino acid sequence without loss or significant loss of a desired activity. The present invention includes such polypeptides with alternate amino acid sequences, and the amino acid sequences encoded by the DNA sequences shown herein merely illustrate preferred embodiments of the invention.

The recombinant nucleic acids, proteins, and peptides of the invention are many and diverse. To facilitate an understanding of the invention and the diverse compounds and methods provided thereby, the following description of the various regions of the megalomicin PKS and the megalomicin modification

enzymes and corresponding coding sequences is provided. To facilitate description of the invention, reference to a PKS, protein, module, or domain herein can also refer to DNA compounds comprising coding sequences therefor and *vice versa*. Also, unless otherwise indicated, reference to a heterologous PKS refers to a PKS or DNA compounds comprising coding sequences therefor from an organism other than *Micromonospora megalomicea*. In addition, reference to a PKS or its coding sequence includes reference to any portion thereof.

Thus, the invention provides DNA molecules in isolated (i.e., not pure, but existing in a preparation in an abundance and/or concentration not found in nature) and purified (i.e., substantially free of contaminating materials or substantially free of materials with which the corresponding DNA would be found in nature) form. The DNA molecules of the invention comprise one or more sequences that encode one or more domains (or fragments of such domains) of one or more modules in one or more of the ORFs of the megalomicin PKS and sequences that encode megalomicin modification enzymes from the megalomicin biosynthetic gene cluster. Examples of PKS domains include the KS, AT, DH, KR, ER, ACP, and TE domains of at least one of the 6 extender modules and loading module of the three proteins encoded by the three ORFs of the megalomicin PKS gene cluster. Examples of megalomicin modification enzymes include those that synthesize the mycarose, desosamine, and megosamine moieties, those that transfer those sugar moieties to the polyketide 6-dEB, those that hydroxylate the polyketide at C-6 and C-12, and those that acylate the sugar moieties.

In an especially preferred embodiment, the DNA molecule is a recombinant DNA expression vector or plasmid, as described in more detail in the following Section. Generally, such vectors can either replicate in the cytoplasm of the host cell or integrate into the chromosomal DNA of the host cell. In either case, the vector can be a stable vector (i.e., the vector remains present over many cell divisions, even if only with selective pressure) or a transient vector (i.e., the vector is gradually lost by host cells with increasing numbers of cell divisions).

The megalomicin PKS gene cluster comprises three ORFs (*megAI*, *megAII*, and *megAIII*). Each ORF encodes two extender modules of the PKS; the first ORF also encodes the loading module. Each extender module is composed of at least a KS, an AT, and an ACP domain. The locations of the various encoding regions of

these ORFs are shown in Figure 2 and described with reference to the sequence information below. The megalomicin PKS produces the polyketide known as 6-dEB, shown in Figure 4. In megalomicin-producing organisms, 6-dEB is converted to erythromycin C by a set of modification enzymes. Thus, 6-dEB is converted to erythronolide B by the *megF* gene product (a homolog of the *eryF* gene product), then to 3-alpha-mycarosyl-erythronolide B by the *megBV* gene product (a homolog of the *eryBV* gene product), then to erythromycin D by the *megCIII* gene product (a homolog of the *eryCIII* gene product, then to erythromycin C by the *megK* gene product (a homolog of the *eryK* gene product).

In addition to these modification enzymes, such megalomicin-producing organisms also contain the modification enzymes necessary for the biosynthesis of the desosamine and mycarose moieties that are similarly utilized in erythromycin biosynthesis, as shown in Figure 5. Megalomicin A contains the complete erythromycin C structure, and its biosynthesis additionally involves the formation of L-megosamine (L-rhodosamine) and its attachment to the C-6 hydroxyl (Figures 3 and 5, inset), followed by acylation of the C-3''' and(or) C-4''' hydroxyls as the terminal steps. L-megosamine is the same as *N*-dimethyl-L-daunosamine; the daunosamine genes have been characterized from *Streptomyces peucetius* (see Colombo and Hutchinson, *J. Indust. Microbiol. Biotechnol.*, in press; Otten *et al.*, 1996, *J Bacteriol* 178:7316-7321, and references cited therein). Some of the rhodosamine genes also have been cloned and partially characterized from another anthracycline producing *Streptomyces* sp. (see Torkkell *et al.*, 1997, *Mol. Gen. Genet.* *256*(2):203-209). Because the timing of the glycosylation with TDP-megosamine in relation to the addition of mycarose and desosamine to erythronolide B, plus the C-12 hydroxylation, is unknown, the pathway could involve a different order of glycosylation and C-12 hydroxylation steps than the one shown in Figure 5. Regardless, the megalomicin biosynthetic gene cluster contains the genes to make L-rhodosamine and attach it to the correct macrolide substrate.

The biosynthetic pathways to make the glycosides desosamine, mycarose, and megosamine are shown in Figure 6. The present invention provides the genes for each biosynthetic pathway shown in this Figure, and these recombinant genetic

pathways can be used alone or in any combination to confer the pathway to a heterologous host.

The megalomicin PKS locus is similar to the *eryA* locus in size and organization. Most of the deoxysugar biosynthesis genes are homologs of the *eryB* mycarose and *eryC* desosamine biosynthesis and glycosyl attachment genes from *Saccharopolyspora erythraea* (see Summers *et al.*, 1997, *Microbiol. 143*:3251-3262; Haydock *et al.*, 1991, *Mol. Gen. Genet.* 230:120-128; Gaisser *et al.*, 1997, *Mol Gen Genet*, 256:239-251; Gaisser *et al.*, 1998, *Mol Gen Genet.* 257:78-88, incorporated herein by reference) or the *picC* homologs from the picromycin and narbomycin producer (see PCT patent publication No. 99/61599 and Xue et al., 1998, *Proc. Nat. Acad. Sci. USA 95*, 12111-12116, incorporated herein by reference). The TDP-megosamine biosynthesis genes are homologs of the *dnm* genes (see Figure 5) and the pikromycin N-dimethyltransferase gene or its homologs reported in a cluster of L-rhodosamine biosynthesis genes. The putative TDP-megosamine glycosyltransferase gene product (*geneX* in Figure 5) closely resembles the deduced products of the *eryBV* , *eryCIII*, *dnmS*, and pikromycin *desVII* genes, even though it recognizes different substrates than the products of each of these genes.

The following Table 1 shows the location of the genes in the *Micromonospora megalomicea* megalomicin biosynthetic pathway in the DNA sequence set forth in SEQ ID NO:1 (see also Figure 7; note some gene designations maybe different in Figure 7).

Table 1. Megalomicin Biosynthetic Gene Cluster
Micromonospora megalomicea subsp. nigra (ATCC27598)

Location	Description
1..2451	sequence from cosmid pKOS079-138B
complement(1..144)	*megBVI (or megT)*, TDP-4-keto-6-deoxyglucose-2,3-dehydratase
928..2061	*megDVI*, TDP-4-keto-6-deoxyglucose 3,4-isomerase
2072..3382	*megDI*, TDP-megosaminyl transferase (*eryCIII* homolog)
2452..40397	sequence of cosmid pKOS079-93D
3462..4634	*megG(or megY)*, mycarosyl acyltransferase
4651..5775	*megDII*, deoxysugar transaminase (*eryCI, DnrJ* homolog)

5822..6595	*megDIII*, TDP-daunosaminyl-N,N-dimethyltransferase (*eryCVI* homolog)
6592..7197	*megDIV*, TDP-4-keto-6-deoxyglucose 3,5-epimerase (*eryBVII*, *dnmU* homolog)
7220..8206	*megDV*, TDP-hexose 4-ketoreductase (*eryBIV*, *dnmV* homolog)
complement(8228..9220)	*megBII*-1 or *megDVII*, TDP-4-keto-L-6-deoxy-hexose 2,3-reductase
complement(9226..10479)	*megBV*, TDP-mycarosyl transferase
complement(10483..11424)	*megBIV*, TDP-hexose 4-ketoreductase
12181..22821	*megAI*
12181..13791	Loading Module (L)
12505..13470	AT-L
13576..13791	ACP-L
13849..18207	Extender Module 1 (1)
13849..15126	KS1
15427..16476	AT1
17155..17694	KR1
17947..18207	ACP1
18268..22575	Extender Module 2 (2)
18268..19548	KS2
19876..20910	AT2
21517..22053	KR2
22318..22575	ACP2
22867..33555	*megAII*
22957..27258	Extender Module 3 (3)
22957..24237	KS3
24544..25581	AT3
26230..26733	KR3 (inactive)
26998..27258	ACP3
27313..33312	Extender Module 4 (4)
27393..28590	KS4
28897..29931	AT4
29953..30477	DH4
31396..32244	ER4
32257..32799	KR4
33052..33312	ACP4
33666..43271	*megAIII*
33780..38120	Extender Module 5 (5)
33780..35027	KS5
35385..36419	AT5
37068..37604	KR5
37860..38120	ACP5
38187..42425	Extender Module 6 (6)
38187..39470	KS6
39795..40811	AT6
40398..46641	sequences from cosmid pKOS079-93A

41406..41936	KR6
42168..42425	ACP6
42585..43271	TE
43268..44344	*megCII*, TDP-4-keto-6-deoxyglucose 3,4-isomerase
44355..45623	*megCIII*, TDP-desosaminyl transferase
45620..46591	*megBII*, TDP-4-keto-6-deoxy-L-glucose 2,3 dehydratase
complement(46660..47403)	*megH*, TEII
complement(47411..47980)	*megF*, C-6 hydroxylase

In a specific embodiment, the invention provides an isolated nucleic acid fragment comprising a nucleotide sequence encoding a domain of the megalomicin polyketide synthase or a megalomicin modification enzyme. The isolated nucleic acid fragment can be a DNA or a RNA. Preferably, the isolated nucleic acid fragment is a recombinant DNA compound. A nucleotide sequence that is complementary to the nucleotide sequence encoding a domain of megalomicin PKS or a megalomicin modification enzyme is also provided.

The isolated nucleic acid fragment can comprise a single, multiple or all the open reading frame(s) (ORF) of the megalomicin PKS or the megalomicin modification enzyme. Exemplary ORFs of megalomicin PKS include the ORFs of the *megAI*, *megAII* and *megAIII* genes. The isolated nucleic acids of the invention also include nucleic acids that encode one or more domains and one or more modules of the megalomicin PKS. Exemplary domains of the megalomicin PKS include a TE domain, a KS domain, an AT domain, an ACP domain, a KR domain, a DH domain and an ER domain. In a preferred embodiment, the nucleic acid comprises the coding sequence for a loading module, a thioesterase domain, and all six extender modules of the megalomicin PKS.

Megalomicin modification enzymes include those enzymes involved in the conversion of 6-DEB into a megalomicin such as the enzymes encoded by *megF*, *meg BV*, *megCIII*, *megK*, *megDI* and *megG* (or *megY*). Megalomicin modification enzymes also include those enzymes involved in the biosynthesis of mycarose, megosamine or desosamine, which are used as biosynthetic intermediates in the biosynthesis of various megalomicin species and other related polyketides. The enzymes that are involved in biosynthesis of mycarose, megosamine or desosamine are described in Figures 5 and 10. The megalomicin PKS and megalomicin modification enzymes are collectively referred to as megalomicin

biosynthetic enzymes; the genes encoding such enzymes are collectively referred to as megalomicin biosynthetic genes; and nucleic acids that comprise a portion of or entire megalomicin biosynthetic genes are collectively referred to as megalomicin biosynthetic nucleic acid(s).

In specific embodiments, the megalomicin biosynthetic nucleic acids comprise the sequence of SEQ ID NO:1, or the coding regions thereof, or nucleotide sequences encoding, in whole or in part, a megalomicin biosynthetic enzyme protein. The isolated nucleic acids typically consists of at least 25 (continuous) nucleotides, 50 nucleotides, 100 nucleotides, 150 nucleotides, or 200 nucleotides of megalomicin biosynthetic nucleic acid sequence, or a full-length megalomicin biosynthetic coding sequence. In another embodiment, the nucleic acids are smaller than 35, 200, or 500 nucleotides in length. Nucleic acids can be single or double stranded. Nucleic acids that hybridize to or are complementary to the foregoing sequences, in particular the inverse complement to nucleic acids that hybridize to the foregoing sequences (*i.e.*, the inverse complement of a nucleic acid strand has the complementary sequence running in reverse orientation to the strand so that the inverse complement would hybridize without mismatches to the nucleic acid strand) are also provided. In specific aspects, nucleic acids are provided which comprise a sequence complementary to (specifically are the inverse complement of) at least 10, 25, 50, 100, or 200 nucleotides or the entire coding region of a megalomicin biosynthetic gene.

The megalomicin biosynthetic nucleic acids provided herein include those with nucleotide sequences encoding substantially the same amino acid sequences as found in native megalomicin biosynthetic enzyme proteins, and those encoding amino acid sequences with functionally equivalent amino acids, as well as megalomicin biosynthetic enzyme derivatives or analogs as described in Section IV.

Some regions within the megalomicin PKS genes are highly homologous or identical to one another, as can be readily identified by an analysis of the sequence. The coding sequence for the KS and AT domains of module 2 shares significant identity with the coding sequence for the KS and AT domains of module 6. This sequence homology or identity at the nucleic acid, *e.g.*, DNA, level can render the nucleic acid unstable in certain host cells. To improve the stability

of the nucleic acids comprising a portion or the entire megalomicin PKS genes and megalomicin modification enzyme genes, the nucleic acid or DNA sequences can be changed to reduce or abolish the sequence homology or identity. Preferably, the DNA codons of homologous regions within the PKS or the megalomicin modification enzyme coding sequence are changed to reduce or abolish the sequence homology or identity without changing the amino acid sequences encoded by said changed DNA codons (see the examples below). The stability of the nucleic acid or DNA can also be improved by codon changes that reduce or abolish the sequence homology or identity while also changing the amino acid sequence, provided that the amino acid sequence change(s) does not substantially change the desired activity of the encoded megalomicin PKS. Thus, for example, one can simply substitute for the *megAIII* ORF an ORF from *eryAIII, oleAIII, picAIII*, or *picAIV* genes.

The recombinant DNA compounds of the invention that encode the megalomicin PKS and modification proteins or portions thereof are useful in a variety of applications. While many of these applications relate to the heterologous expression of the megalomicin biosynthetic genes or the construction of hybrid PKS enzymes, many useful applications involve the natural megalomicin producer *Micromonospora megalomicea*. For example, one can use the recombinant DNA compounds of the invention to disrupt the megalomicin biosynthetic genes by homologous recombination in *Micromonospora megalomicea*. The resulting host cell is a preferred host cell for making polyketides modified by oxidation, hydroxylation, glycosylation, and acylation in a manner similar to megalomicin, because the genes that encode the proteins that perform these reactions are of course present in the host cell, and because the host cell does not produce megalomicin that could interfere with production or purification of the polyketide of interest.

One illustrative recombinant host cell provided by the present invention expresses a recombinant megalomicin PKS in which the module 1 KS domain is inactivated by deletion or other mutation. In a preferred embodiment, the inactivation is mediated by a change in the KS domain that renders it incapable of binding substrate (called a KS1° mutation). In a particularly preferred embodiment, this inactivation is rendered by a mutation in the codon for the active

site cysteine that changes the codon to another codon, such as an alanine codon. Such constructs are especially useful when placed in translational reading frame with extender modules 1 and 2 of a megalomicin or the corresponding modules of another PKS. The utility of these constructs is that host cells expressing, or cell free extracts containing, a PKS comprising the protein encoded thereby can be fed or supplied with N-acylcysteamine thioesters of precursor molecules to prepare a polyketide of interest. See U.S. patent application Serial No. 09/492,773, filed 27 Jan. 2000, and PCT patent publication No. 00/44717, both of which are incorporated herein by reference. Such KS1° constructs of the invention are useful in the production of 13-substituted-megalomicin compounds in *Micromonospora megalomicea* host cells. Preferred compounds of the invention include those compounds in which the substituent at the 13-position is propyl, vinyl, propargyl, other lower alkyl, and substituted alkyl.

In a variant of this embodiment, one can employ a megalomicin PKS in which the ACP domain of module 1 has been rendered inactive. In another embodiment, one can delete the loading domain of the megalomicin PKS and provide monoketide substrates for processing by the remainder of the PKS.

The compounds of the invention can also be used to construct recombinant host cells of the invention in which coding sequences for one or more domains or modules of the megalomicin PKS or for another megalomicin biosynthetic gene have been deleted by homologous recombination with the *Micromonospora megalomicea* chromosomal DNA. Those of skill in the art will appreciate that the compounds used in the recombination process are characterized by their homology with the chromosomal DNA and not by encoding a functional protein due to their intended function of deleting or otherwise altering portions of chromosomal DNA. For this and a variety of other applications, the compounds of the present invention include not only those DNA compounds that encode functional proteins but also those DNA compounds that are complementary or identical to any portion of the megalomicin biosynthetic genes.

Thus, the invention provides a variety of modified *Micromonospora megalomicea* host cells in which one or more of the megalomicin biosynthetic genes have been mutated or disrupted. Transformation systems for *M. megalomicea* have been described by Hasegawa *et al.*, 1991, *J. Bacteriol.*

173:7004-11; and Takada *et al.*, 1994, *J. Antibiot.* *47*:1167-1170, both of which are incorporated herein by reference. These cells are especially useful when it is desired to replace the disrupted function with a gene product expressed by a recombinant DNA expression vector. While such expression vectors of the invention are described in more detail in the following Section, those of skill in the art will appreciate that the vectors have application to *M. megalomicea* as well. Such *M. megalomicea* host cells can be preferred host cells for expressing megalomicin derivatives of the invention. Particularly preferred host cells of this type include those in which the coding sequence for the loading module has been mutated or disrupted, those in which one or more of any of the PKS gene ORFs has been mutated or disrupted, and/or those in which the genes for one or more modification (glycosylation, acylation, hydroxylation) have been mutated or disrupted.

While the present invention provides many useful compounds having application to, and recombinant host cells derived from, *Micromonospora megalomicea*, many important applications of the present invention relate to the heterologous expression of all or a portion of the megalomicin biosynthetic genes in cells other than *M. megalomicea*, as described in Section V.

<u>Section IV: The Megalomicin Biosynthetic Enzymes and Antibodies Recognizing such Enzymes</u>

In another specific embodiment, the invention provides a substantially purified polypeptide, which is encoded by a nucleic acid fragment comprising a nucleotide sequence encoding a domain of megalomicin polyketide synthase (PKS) or a megalomicin modification enzyme. The polypeptide can comprise a single domain, multiple domains or a full-length megalomicin PKS or megalomicin modification enzyme. Functional fragments, analogs or derivatives of the megalomicin PKS or megalomicin modification enzyme polypeptides are also provided. Preferably, such fragments, analogs or derivatives can be recognized an antibody raised against a megalomicin PKS or megalomicin modification enzyme. Also preferably, such fragments, analogs or derivatives comprise an amino acid sequence that has at least 60% identity, more preferably at least 90% identity to their wild type counterparts.

An exemplary nucleotide sequence encoding, and the corresponding amino acid sequence of, a megalomicin biosynthetic enzyme is disclosed in SEQ ID NO:1. Homologs (*e.g.*, nucleic acids of the above-listed genes of species other than *Micromonospora megalomicea*) or other related sequences (*e.g.*, paralogs) can be obtained by low, moderate or high stringency hybridization with all or a portion of the particular sequence provided as a probe using methods well known in the art for nucleic acid hybridization and cloning (*e.g.*, as described in Section III) in accordance with the methods of the present invention.

The megalomicin biosynthetic enzyme proteins, or domains thereof, of the present invention can be obtained by methods well known in the art for protein purification and recombinant protein expression in accordance with the methods of the present invention. For recombinant expression of one or more of the proteins, the nucleic acid containing all or a portion of the nucleotide sequence encoding the protein can be inserted into an appropriate expression vector, *i.e.*, a vector that contains the necessary elements for the transcription and translation of the inserted protein coding sequence. Transcriptional and translational signals can be supplied by the native promoter for a megalomicin biosynthetic gene and/or flanking regions.

A variety of host-vector systems may be utilized to express the protein coding sequence. These include but are not limited to mammalian cell systems infected with virus (*e.g.* vaccinia virus, adenovirus, and the like); insect cell systems infected with virus (*e.g.* baculovirus); microorganisms such as yeast containing yeast vectors; or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their properties. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used.

In a specific embodiment, a vector is used that comprises a promoter operably linked to nucleic acid sequences encoding a megalomicin biosynthetic enzyme, or a domain, fragment, derivative or homolog, thereof, one or more origins of replication, and optionally, one or more selectable markers (*e.g.*, an antibiotic resistance gene).

Expression vectors containing the sequences of interest can be identified by three general approaches: (a) nucleic acid hybridization, (b) presence or

absence of "marker" gene function, and (c) expression of the inserted sequences. In the first approach, megalomicin biosynthetic nucleic acid sequences can be detected by nucleic acid hybridization to probes comprising sequences homologous and complementary to the inserted sequences. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" functions (*e.g.*, binding to an anti-megalomicin biosynthetic enzyme antibody, resistance to antibiotics, occlusion body formation in baculovirus, and the like) caused by insertion of the sequences of interest in the vector. For example, if a megalomicin biosynthetic gene, or portion thereof, is inserted within the marker gene sequence of the vector, recombinants containing the megalomicin biosynthetic gene fragment will be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying for the megalomicin biosynthetic gene products expressed by the recombinant vector. Such assays can be based, for example, on the physical or functional properties of the interacting species in *in vitro* assay systems, *e.g.*, megalomicin synthesis activity, immunoreactivity to antibodies specific for the protein.

Once recombinant megalomicin biosynthetic genes or nucleic acids are identified, several methods known in the art can be used to propagate them in accordance with the methods of the present invention. Once a suitable host system and growth conditions have been established, recombinant expression vectors can be propagated and amplified in quantity. As previously described, the expression vectors or derivatives which can be used include, but are not limited to: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus, yeast vectors; bacteriophage vectors such as lambda phage; and plasmid and cosmid vectors.

In addition, a host cell strain may be chosen that modulates the expression of the inserted sequences, or modifies or processes the expressed proteins in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus expression of the genetically-engineered megalomicin biosynthetic enzymes may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (*e.g.* glycosylation, phosphorylation, and

the like) of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein is achieved. For example, expression in a bacterial system can be used to produce an unglycosylated core protein, while expression in mammalian cells ensures "native" glycosylation of a heterologous protein. Furthermore, different vector/host expression systems may effect processing reactions to different extent.

In particular, megalomicin biosynthetic enzyme derivatives can be made by altering their sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as an megalomicin biosynthetic gene can be used in the practice of the present invention. These include but are not limited to nucleotide sequences comprising all or portions of megalomicin biosynthetic genes that are altered by the substitution of different codons that encode the amino acid residue within the sequence, thus producing a silent change. Likewise, the megalomicin biosynthetic enzyme derivatives of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of megalomicin biosynthetic enzymes, including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

In a specific embodiment of the invention, the nucleic acids encoding proteins and proteins consisting of or comprising a domain or a fragment of megalomicin biosynthetic enzyme consisting of at least 6 (continuous) amino

acids are provided. In other embodiments, the domain or fragment consists of at least 10, 20, 30, 40, or 50 amino acids of a megalomicin biosynthetic enzyme. In specific embodiments, such domains or fragments are not larger than 35, 100 or 200 amino acids. Derivatives or analogs of megalomicin biosynthetic enzyme include but are not limited to molecules comprising regions that are substantially homologous to megalomicin biosynthetic enzyme in various embodiments, at least 30%, 40%, 50%, 60%, 70%, 80%, 90% or 95% identity over an amino acid sequence of identical size or when compared to an aligned sequence in which the alignment is done by a computer homology program known in the art in accordance with the methods of the present invention or whose encoding nucleic acid is capable of hybridizing to a sequence encoding a megalomicin biosynthetic enzyme under stringent, moderately stringent, or nonstringent conditions.

The megalomicin biosynthetic enzyme domains, derivatives and analogs of the invention can be produced by various methods known in the art in accordance with the methods of the present invention. The manipulations which result in their production can occur at the gene or protein level. For example, the cloned megalomicin biosynthetic gene sequence can be modified by any of numerous strategies known in the art (Sambrook et al., 1990, *Molecular Cloning, A Laboratory Manual,* 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) in accordance with the methods of the present invention. The sequences can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*.

Additionally, the megalomicin biosynthetic enzyme-encoding nucleotide sequence can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy pre-existing ones, to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art can be used in accordance with the methods of the present invention, including but not limited to, chemical mutagenesis and *in vitro* site-directed mutagenesis (Hutchinson et al., *J. Biol. Chem.* 253:6551-6558 (1978)), use of TAB® linkers (Pharmacia), and the like.

Once a recombinant cell expressing a megalomicin biosynthetic enzyme protein, or a domain, fragment or derivative thereof, is identified, the individual gene product can be isolated and analyzed. This is achieved by assays based on the physical and/or functional properties of the protein, including, but not limited to, radioactive labeling of the product followed by analysis by gel electrophoresis, immunoassay, cross-linking to marker-labeled product, and the like.

The megalomicin biosynthetic enzyme proteins may be isolated and purified by standard methods known in the art or recombinant host cells expressing the complexes or proteins in accordance with the methods of the invention, including but not restricted to column chromatography (*e.g.*, ion exchange, affinity, gel exclusion, reversed-phase high pressure, fast protein liquid, and the like), differential centrifugation, differential solubility, or by any other standard technique used for the purification of proteins. Functional properties may be evaluated using any suitable assay known in the art in accordance with the methods of the present invention.

Alternatively, once a megalomicin biosynthetic enzyme or its domain or derivative is identified, the amino acid sequence of the protein can be deduced from the nucleotide sequence of the gene which encodes it. As a result, the protein or its domain or derivative can be synthesized by standard chemical methods known in the art in accordance with the methods of the present invention (see Hunkapiller et al, *Nature* 310:105-111 (1984)).

Manipulations of megalomicin biosynthetic enzymes may be made at the protein level. Included within the scope of the invention are megalomicin biosynthetic enzyme domains, derivatives or analogs or fragments, which are differentially modified during or after translation, *e.g.*, by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, and the like. Any of numerous chemical modifications may be carried out by known techniques, including but not limited to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, $NaBH_4$, acetylation, formylation, oxidation, reduction, metabolic synthesis in the presence of tunicamycin, and the like.

In specific embodiments, the megalomicin biosynthetic enzymes are modified to include a fluorescent label. In other specific embodiments, the megalomicin biosynthetic enzyme is modified to have a heterofunctional reagent, such heterofunctional reagents can be used to crosslink the members of the complex.

In addition, domains, analogs and derivatives of a megalomicin biosynthetic enzyme can be chemically synthesized. For example, a peptide corresponding to a portion of a megalomicin biosynthetic enzyme, which comprises the desired domain or which mediates the desired activity *in vitro* can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the megalomicin biosynthetic enzyme sequence. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, alpha-amino isobutyric acid, 4-aminobutyric acid, 2-aminobutyric acid, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionoic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, ß-alanine, fluoro-amino acids, designer amino acids such as ß-methyl amino acids, Ca-methyl amino acids, Na-methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

In cases where natural products are suspected of being mutant or are isolated from new species, the amino acid sequence of the megalomicin biosynthetic enzyme isolated from the natural source, as well as those expressed *in vitro*, or from synthesized expression vectors *in vivo* or *in vitro*, can be determined from analysis of the DNA sequence, or alternatively, by direct sequencing of the isolated protein. Such analysis may be performed by manual sequencing or through use of an automated amino acid sequenator.

The megalomicin biosynthetic enzyme proteins may also be analyzed by hydrophilicity analysis (Hopp and Woods, *Proc. Natl. Acad. Sci. USA* 78:3824-3828 (1981)). A hydrophilicity profile can be used to identify the hydrophobic and hydrophilic regions of the proteins, and help predict their orientation in designing substrates for experimental manipulation, such as in binding

experiments, antibody synthesis, and the like. Secondary structural analysis can also be done to identify regions of the megalomicin biosynthetic enzyme that assume specific structures (Chou and Fasman, *Biochemistry* 13:222-23 (1974)). Manipulation, translation, secondary structure prediction, hydrophilicity and hydrophobicity profiles, open reading frame prediction and plotting, and determination of sequence homologies, can be accomplished using computer software programs available in the art.

Other methods of structural analysis including but not limited to X-ray crystallography (Engstrom, *Biochem. Exp. Biol.* 11:7-13 (1974)), mass spectroscopy and gas chromatography (Methods in Protein Science, J. Wiley and Sons, New York, 1997), and computer modeling (Fletteriek and Zoller, eds., 1986, Computer Graphics and Molecular Modeling, In: *Current Communications in Molecular Biology,* Cold Spring Harbor Laboratory, Cold Spring Harbor Press, New York) can also be employed.

The invention also provides an antibody, or a fragment or derivative thereof, which immuno-specifically binds to a domain of megalomicin polyketide synthase (PKS) or a megalomicin modification enzyme. In a specific embodiment, an antibody which immuno-specifically binds to a domain of the megalomicin biosynthetic enzyme encoded by a nucleic acid that hybridizes to a nucleic acid having the nucleotide sequence set forth in the SEQ. ID NO:1, or a fragment or derivative of said antibody containing the binding domain thereof is provided. Preferably, the antibody is a monoclonal antibody.

The megalomicin biosynthetic enzyme protein and domains, fragments, homologs and derivatives thereof may be used as immunogens to generate antibodies which immunospecifically bind such immunogens. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library.

Various procedures known in the art may be used for the production of polyclonal antibodies to a megalomicin biosynthetic enzyme protein of the invention, its domains, derivatives, fragments or analogs in accordance with the methods of the present invention.

For production of the antibody, various host animals can be immunized by injection with the native megalomicin biosynthetic enzyme protein or a synthetic

version, or a derivative of the foregoing, such as a cross-linked megalomicin biosynthetic enzyme. Such host animals include but are not limited to rabbits, mice, rats, and the like. Various adjuvants can be used to increase the immunological response, depending on the host species, and include but are not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, dinitrophenol, and potentially useful human adjuvants such as bacille Calmette-Guerin (BCG) and corynebacterium parvum.

For preparation of monoclonal antibodies directed towards a megalomicin biosynthetic enzyme or domains, derivatives, fragments or analogs thereof, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used. Such techniques include but are not restricted to the hybridoma technique originally developed by Kohler and Milstein (*Nature* 256:495-497 (1975)), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., *Immunology Today* 4:72 (1983)), and the EBV hybridoma technique to produce human monoclonal antibodies (Cole et al., *in Monoclonal Antibodies and Cancer Therapy,* Alan R. Liss, Inc., pp. 77-96 (1985)). In an additional embodiment, monoclonal antibodies can be produced in germ-free animals (WO89/12690). Human antibodies may be used and can be obtained by using human hybridomas (Cote et al., *Proc. Natl. Acad. Sci. USA* 80:2026-2030 (1983)) or by transforming human B cells with EBV virus *in vitro* (Cole et al., *in Monoclonal Antibodies and Cancer Therapy,* Alan R. Liss, Inc., pp. 77-96 (1985)). Techniques developed for the production of "chimeric antibodies" (Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984); Neuberger et al., *Nature* 312:604-608 (1984); Takeda et al., *Nature* 314:452-454 (1985)) by splicing the genes from a mouse antibody molecule specific for the megalomicin biosynthetic enzyme protein together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention.

Techniques described for the production of single chain antibodies (U.S. patent 4,946,778) can be adapted to produce megalomicin biosynthetic enzyme-specific single chain antibodies. An additional embodiment utilizes the techniques described for the construction of Fab expression libraries (Huse et al., *Science*

246:1275-1281 (1989)) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for megalomicin biosynthetic enzyme, or domains, derivatives, or analogs thereof. Non-human antibodies can be "humanized" by known methods (*see, e.g.*, U.S. Patent No. 5,225,539).

Antibody fragments that contain the idiotypes of a megalomicin biosynthetic enzyme can be generated by techniques known in the art in accordance with the methods of the present invention. For example, such fragments include but are not limited to: the F(ab')2 fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments that can be generated by reducing the disulfide bridges of the F(ab')2 fragment, the Fab fragments that can be generated by treating the antibody molecular with papain and a reducing agent, and Fv fragments.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art in accordance with the methods of the present invention, *e.g.*, ELISA (enzyme-linked immunosorbent assay). To select antibodies specific to a particular domain of the megalomicin biosynthetic enzyme, one may assay generated hybridomas for a product that binds to the fragment of a megalomicin biosynthetic enzyme that contains such a domain.

The foregoing antibodies can be used in methods known in the art relating to the localization and/or quantitation of megalomicin biosynthetic enzyme proteins, *e.g.*, for imaging these proteins or measuring levels thereof in samples, in accordance with the methods of the present invention.

Section V: Heterologous Expression of the Megalomicin Biosynthetic Genes

In one important embodiment, the invention provides methods for the heterologous expression of one or more of the megalomicin biosynthetic genes and recombinant DNA expression vectors useful in the method. For purposes of the invention, any host cell other than *Micromonospora megalomicea* is a heterologous host cell. Thus, included within the scope of the invention in addition to isolated nucleic acids encoding domains, modules, or proteins of the megalomicin PKS and modification enzymes, are recombinant expression vectors that include such nucleic acids. The term expression vector refers to a nucleic acid that can be introduced into a host cell or cell-free transcription and translation

system. An expression vector can be maintained permanently or transiently in a cell, whether as part of the chromosomal or other DNA in the cell or in any cellular compartment, such as a replicating vector in the cytoplasm. An expression vector also comprises a promoter that drives expression of an RNA, which typically is translated into a polypeptide in the cell or cell extract. For efficient translation of RNA into protein, the expression vector also typically contains a ribosome-binding site sequence positioned upstream of the start codon of the coding sequence of the gene to be expressed. Other elements, such as enhancers, secretion signal sequences, transcription termination sequences, and one or more marker genes by which host cells containing the vector can be identified and/or selected, may also be present in an expression vector. Selectable markers, i.e., genes that confer antibiotic resistance or sensitivity, are preferred and confer a selectable phenotype on transformed cells when the cells are grown in an appropriate selective medium.

The various components of an expression vector can vary widely, depending on the intended use of the vector and the host cell(s) in which the vector is intended to replicate or drive expression. Expression vector components suitable for the expression of genes and maintenance of vectors in *E. coli*, yeast, *Streptomyces*, and other commonly used cells are widely known and commercially available. For example, suitable promoters for inclusion in the expression vectors of the invention include those that function in eucaryotic or procaryotic host cells. Promoters can comprise regulatory sequences that allow for regulation of expression relative to the growth of the host cell or that cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus. For *E. coli* and certain other bacterial host cells, promoters derived from genes for biosynthetic enzymes, antibiotic-resistance conferring enzymes, and phage proteins can be used and include, for example, the galactose, lactose (*lac*), maltose, tryptophan (*trp*), beta-lactamase (*bla*), bacteriophage lambda PL, and T5 promoters. In addition, synthetic promoters, such as the *tac* promoter (U.S. Patent No. 4,551,433), can also be used.

Thus, recombinant expression vectors contain at least one expression system, which, in turn, is composed of at least a portion of the megalomicin PKS and/or other megalomicin biosynthetic gene coding sequences operably linked to a

promoter and optionally termination sequences that operate to effect expression of the coding sequence in compatible host cells. The host cells are modified by transformation with the recombinant DNA expression vectors of the invention to contain the expression system sequences either as extrachromosomal elements or integrated into the chromosome. The resulting host cells of the invention are useful in methods to produce PKS and post-PKS modification enzymes as well as polyketides and antibiotics and other useful compounds derived therefrom.

Preferred host cells for purposes of selecting vector components for expression vectors of the present invention include fungal host cells such as yeast and procaryotic host cells such as *E. coli* and *Streptomyces*, but mammalian host cells can also be used. In hosts such as yeasts, plants, or mammalian cells that ordinarily do not produce polyketides, it may be necessary to provide, also typically by recombinant means, suitable holo-ACP synthases to convert the recombinantly produced PKS to functionality. Provision of such enzymes is described, for example, in PCT publication Nos. WO 97/13845 and 98/27203, each of which is incorporated herein by reference. Particularly preferred host cells for purposes of the present invention are *Streptomyces* and *Saccharopolyspora* host cells, as discussed in greater detail below.

In a preferred embodiment, the expression vectors of the invention are used to construct a heterologous recombinant *Streptomyces* host cell that expresses a recombinant PKS of the invention. *Streptomyces* is a convenient host for expressing polyketides, because polyketides are naturally produced in certain *Streptomyces* species, and *Streptomyces* cells generally produce the precursors needed to form the desired polyketide. Those of skill in the art will recognize that, if a *Streptomyces* host cell produces any portion of a PKS enzyme or produces a polyketide modification enzyme, the recombinant vector need drive expression of only those genes constituting the remainder of the desired PKS enzyme or other polyketide-modifying enzymes. Thus, such a vector may comprise only a single ORF, with the desired remainder of the polypeptides constituting the PKS provided by the genes on the host cell chromosomal DNA.

If a *Streptomyces* or other host cell ordinarily produces polyketides, it may be desirable to modify the host so as to prevent the production of endogenous polyketides prior to its use to express a recombinant PKS of the invention. Such

modified hosts include *S. coelicolor* CH999 and similarly modified *S. lividans* described in U.S. Patent No. 5,672,491, and PCT publication Nos. WO 95/08548 and WO 96/40968, incorporated herein by reference. In such hosts, it may not be necessary to provide enzymatic activities for all of the desired post-translational modifications of the enzymes that make up the recombinantly produced PKS, because the host naturally expresses such enzymes. In particular, these hosts generally contain holo-ACP synthases that provide the phosphopantotheinyl residue needed for functionality of the PKS.

The invention provides a wide variety of expression vectors for use in *Streptomyces*. The replicating expression vectors of the present invention include, for example and without limitation, those that comprise an origin of replication from a low copy number vector, such as SCP2* (see Hopwood *et al.*, *Genetic Manipulation of Streptomyces: A Laboratory manual* (The John Innes Foundation, Norwich, U.K., 1985); Lydiate *et al.*, 1985, *Gene 35*: 223-235; and Kieser and Melton, 1988, *Gene 65*: 83-91, each of which is incorporated herein by reference), SLP1.2 (Thompson *et al.*, 1982, *Gene 20*: 51-62, incorporated herein by reference), and pSG5(ts) (Muth *et al.*, 1989, *Mol. Gen. Genet. 219*: 341-348, and Bierman *et al.*, 1992, *Gene 116*: 43-49, each of which is incorporated herein by reference), or a high copy number vector, such as pIJ101 and pJV1 (see Katz *et al.*, 1983, *J. Gen. Microbiol. 129*: 2703-2714; Vara *et al.*, 1989, *J. Bacteriol. 171*: 5782-5781; and Servin-Gonzalez, 1993, *Plasmid 30*: 131-140, each of which is incorporated herein by reference). For non-replicating and integrating vectors and generally for any vector, it is useful to include at least an *E. coli* origin of replication, such as from pUC, p1P, p1I, and pBR. For phage based vectors, the phage phiC31 and its derivative KC515 can be employed (see Hopwood *et al.*, *supra*). Also, plasmid pSET152, plasmid pSAM, plasmids pSE101 and pSE211, all of which integrate site-specifically in the chromosomal DNA of *S. lividans*, can be employed for purposes of the present invention.

The *Streptomyces* recombinant expression vectors of the invention typically comprise one or more selectable markers, including antibiotic resistance conferring genes selected from the group consisting of the *ermE* (confers resistance to erythromycin and lincomycin), *tsr* (confers resistance to thiostrepton), *aadA* (confers resistance to spectinomycin and streptomycin), *aacC4*

(confers resistance to apramycin, kanamycin, gentamicin, geneticin (G418), and neomycin), *hyg* (confers resistance to hygromycin), and *vph* (confers resistance to viomycin) resistance conferring genes. Alternatively, several polyketides are naturally colored, and this characteristic can provide a built-in marker for identifying cells.

Megalomicins are currently produced only by the relatively genetically intractable host *Micromonospora megalomicinea*. This bacteria has not been commonly used in the fermentation industry for the large-scale production of antibiotics, and methods for high level production of megalomicin and its analogs are needed. In contrast, the streptomycete bacteria have been widely used for almost 50 years and are excellent hosts for production of megalomicin and its analogs. *Streptomyces lividans* and *S. coelicolor* have been developed for the expression of heterologous PKS systems. These organisms can stably maintain cloned heterologous PKS genes, express them at high levels under controlled conditions, and modify the corresponding PKS proteins (e.g., phosphopantotheinylation) so that they are capable of production of the polyketide they encode. Furthermore, these hosts contain the necessary pathways to produce the substrates required for polyketide synthesis; e.g. propionyl-CoA and methylmalonyl-CoA. A wide variety of cloning and expression vectors are available for these hosts, as are methods for the introduction and stable maintenance of large segments of foreign DNA. Relative to *Micromonospora* spp., *S. lividans* and *S. coelicolor* grow well on a number of media and have been adapted for high level production of polyketides in fermentors. If production levels are low, a number of rational approaches are available to improve yield (see Hosted and Baltz, 1996, *Trends Biotechnol.* *14*(7):245-50, incorporated herein by reference). Empirical methods to increase the titers of these macrolides, long since proven effective for numerous bacterial polyketides, can also be employed.

Preferred *Streptomyces* host cell/vector combinations of the invention include *S. coelicolor* CH999 and *S. lividans* K4-114 host cells, which have been modified so as not to produce the polyketide actinorhodin, and expression vectors derived from the pRM1 and pRM5 vectors, as described in U.S. Patent Nos. 5,830,750 and 6,022,731 and U.S. patent application Serial No. 09/181,833, filed 28 Oct. 1998, each of which is incorporated herein by reference. These vectors are

particularly preferred in that they contain promoters compatible with numerous and diverse *Streptomyces spp*. Particularly useful promoters for *Streptomyces* host cells include those from PKS gene clusters that result in the production of polyketides as secondary metabolites, including promoters from aromatic (Type II) PKS gene clusters. Examples of Type II PKS gene cluster promoters are *act* gene promoters and *tcm* gene promoters; an example of a Type I PKS gene cluster promoter are the promoters of the spiramycin PKS genes and DEBS genes. The present invention also provides the megalomicin biosynthetic gene promoters in recombinant form. These promoters can be used to drive expression of the megalomicin biosynthetic genes or any other coding sequence of interest in host cells in which the promoter functions, particularly *Micromonospora megalomicea* and generally any *Streptomyces* species.

As described above, particularly useful control sequences are those that alone or together with suitable regulatory systems activate expression during transition from growth to stationary phase in the vegetative mycelium. The promoter contained in the aforementioned plasmid pRM5, i.e., the *actI/actIII* promoter pair and the *actII-ORF4* activator gene, is particularly preferred. Other useful *Streptomyces* promoters include without limitation those from the *ermE* gene and the *melC1* gene, which act constitutively, and the *tipA* gene and the *merA* gene, which can be induced at any growth stage. In addition, the T7 RNA polymerase system has been transferred to *Streptomyces* and can be employed in the vectors and host cells of the invention. In this system, the coding sequence for the T7 RNA polymerase is inserted into a neutral site of the chromosome or in a vector under the control of the inducible *merA* promoter, and the gene of interest is placed under the control of the T7 promoter. As noted above, one or more activator genes can also be employed to enhance the activity of a promoter. Activator genes in addition to the *actII-ORF4* gene described above include *dnrI*, *redD*, and *ptpA* genes (see U.S. patent application Serial No. 09/181,833, supra).

To provide a preferred host cell and vector for purposes of the invention, the megalomicin biosynthetic genes are placed on a recombinant expression vector and transferred to the non-macrolide producing hosts *Streptomyces lividans* K4-114 and *S. coelicolor* CH999. Transformation of *S. lividans* K4-114 or *S. coelicolor* CH999 with this expression vector results in a strain which produces

detectable amounts of megalomicin as determined by analysis of extracts by LC/MS. As noted above, the present invention also provides recombinant DNA compounds in which the encoded megalomicin module 1 KS domain is inactivated (the KS1° mutation). The introduction into *Streptomyces lividans* or *S. coelicolor* of a recombinant expression vector of the invention that encodes a megalomicin PKS with a KS1° domain produces a host cell useful for making polyketides by a process known as diketide feeding. The resulting host cells can be fed or supplied with N-acylcysteamine thioesters of precursor molecules to prepare megalomicin derivatives. Such cells of the invention are especially useful in the production of 13-substituted-6-deoxyerythronolide B compounds in recombinant host cells. Preferred compounds of the invention include those compounds in which the substituent at the 13-position is propyl, vinyl, propargyl, other lower alkyl, and substituted alkyl. In a preferred embodiment, the meg PKS is produced from a recombinant construct in which the *megAIII* gene has been altered to abolish the regions of identical coding sequence it otherwise shares with the *megAI* gene, or a hybrid PKS is employed in which the *megAIII* gene product has been replaced by the *oleAIII* gene product. Recombinant oleAIII genes are described in, for example, PCT patent publication No. 00/026349 and U.S. patent application Serial No. 09/428,517, filed 28 Oct. 1999, both of which are incorporated herein by reference.

The recombinant host cells of the invention can express all of the megalomicin biosynthetic genes or only a subset of the same. For example, if only the genes for the megalomicin PKS are expressed in a host cell that otherwise does not produce polyketide modifying enzymes that can act on the polyketide produced, then the host cell produces unmodified polyketides, called macrolide aglycones. Such macrolide aglycones can be hydroxylated and glycosylated by adding them to the fermentation of a strain such as, for example, *Streptomyces antibioticus* or *Saccharopolyspora erythraea*, that contains the requisite modification enzymes.

There are a wide variety of diverse organisms that can modify macrolide aglycones to provide compounds with, or that can be readily modified to have, useful activities. For example, as shown in Figure 5, *Saccharopolyspora erythraea* can convert 6-dEB to a variety of useful compounds. The erythronolide 6-dEB is

converted by the *eryF* gene product to erythronolide B, which is, in turn, glycosylated by the *eryBV* gene product to obtain 3-O-mycarosylerythronolide B, which contains L-mycarose at C-3. The *eryCIII* gene product then converts this compound to erythromycin D by glycosylation with D-desosamine at C-5. Erythromycin D, therefore, differs from 6-dEB through glycosylation and by the addition of a hydroxyl group at C-6. Erythromycin D can be converted to erythromycin B in a reaction catalyzed by the *eryG* gene product by methylating the L-mycarose residue at C-3. Erythromcyin D is converted to erythromycin C by the addition of a hydroxyl group at C-12 in a reaction catalyzed by the *eryK* gene product. Erythromycin A is obtained from erythromycin C by methylation of the mycarose residue in a reaction catalyzed by the *eryG* gene product. The unmodified megalomicin compounds provided by the present invention, such as, for example, the 6-dEB or 6-dEB analogs, produced in *Streptomyces lividans*, can be provided to cultures of *S. erythraea* and converted to the corresponding derivatives of erythromycins A, B, C, and D in accordance with the procedure provided in the examples below. To ensure that only the desired compound is produced, one can use an *S. erythraea eryA* mutant that is unable to produce 6-dEB but can still carry out the desired conversions (Weber *et al.*, 1985, *J. Bacteriol. 164*(1): 425-433). Also, one can employ other mutant strains, such as *eryB*, *eryC*, *eryG*, and/or *eryK* mutants, or mutant strains having mutations in multiple genes, to accumulate a preferred compound. The conversion can also be carried out in large fermentors for commercial production.

Moreover, there are other useful organisms that can be employed to hydroxylate and/or glycosylate the compounds of the invention. As described above, the organisms can be mutants unable to produce the polyketide normally produced in that organism, the fermentation can be carried out on plates or in large fermentors, and the compounds produced can be chemically altered after fermentation. Thus, *Streptomyces venezuelae*, which produces picromycin, contains enzymes that can transfer a desosaminyl group to the C-5 hydroxyl and a hydroxyl group to the C-12 position. In addition, *S. venezuelae* contains a glucosylation activity that glucosylates the 2'-hydroxyl group of the desosamine sugar. This latter modification reduces antibiotic activity, but the glucosyl residue is removed by enzymatic action prior to release of the polyketide from the cell.

Another organism, *S. narbonensis*, contains the same modification enzymes as *S. venezuelae*, except the C-12 hydroxylase. Thus, the present invention provides the compounds produced by hydroxylation and glycosylation of the macrolide aglycones of the invention by action of the enzymes endogenous to *S. narbonensis* and *S. venezuelae*.

Other organisms suitable for making compounds of the invention include *Micromonospora megalomicea* (discussed above), *Streptomyces antibioticus*, *S. fradiae*, and *S. thermotolerans*. *S. antibioticus* produces oleandomycin and contains enzymes that hydroxylate the C-6 and C-12 positions, glycosylate the C-3 hydroxyl with oleandrose and the C-5 hydroxyl with desosamine, and form an epoxide at C-8-C-8a. *S. fradiae* contains enzymes that glycosylate the C-5 hydroxyl with mycaminose and then the 4'-hydroxyl of mycaminose with mycarose, forming a disaccharide. *S. thermotolerans* contains the same activities as *S. fradiae*, as well as acylation activities. Thus, the present invention provides the compounds produced by hydroxylation and glycosylation of the macrolide aglycones of the invention by action of the enzymes endogenous to *S. antibioticus*, *S. fradiae*, and *S. thermotolerans*.

The present invention also provides methods and genetic constructs for producing the glycosylated and/or hydroxylated compounds of the invention directly in the host cell of interest. Thus, the recombinant genes of the invention, which include recombinant *megAI*, *megAII*, and *megAIII* genes with one or more deletions and/or insertions, including replacements of a *megA* gene fragment with a gene fragment from a heterologous PKS gene (as discussed in the next Section), can be included on expression vectors suitable for expression of the encoded gene products in *Saccharopolyspora erythraea*, *Streptomyces antibioticus*, *S. venezuelae*, *S. narbonensis*, *Micromonospora megalomicea*, *S. fradiae*, and *S. thermotolerans*.

A number of erythromycin high-producing strains of *Saccharopolyspora erythraea* and *Streptomyces fradiae* have been developed, and in a preferred embodiment, the megalomicin PKS and/or other megalomicin biosynthetic genes are introduced into such strains (or erythromycin non-producing mutants thereof) to provide the corresponding modified megalomicin compounds in high yields. Those of skill in the art will appreciate that *S. erythraea* contains the desosamine

and mycarose biosynthetic and transfer genes as well as DEBS, which, as noted above, makes the same macrolide aglycone, 6-dEB, as the megalomicin PKS. *S. erythraea* does not make megosamine or its corresponding transferase gene, and does not contain the acylation gene of *Micromonospora megalomicea*. Finally, the *S. erythraea eryG* gene product converts mycarose to cladinose, which does not occur in *M. megalomicea*. Thus, the present invention provides a wide variety of *S. erythraea* recombinant host cells, including, for example, those that contain:

(i) wild-type erythromycin biosynthetic genes with recombinant megosamine biosynthetic and transfer genes, with and without megalomicin acylation genes;

(ii) wild-type erythromycin biosynthetic genes except *eryG*, with recombinant megosamine biosynthetic and transfer genes, with and without megalomicin acylation genes; and

(iii) as in (i) and (ii), except that the *eryA* genes are inactive or deleted and recombinant *megA* genes have been introduced.

The invention provides other *S. erythraea* strains as well, including those in which any one or more of the erythromycin biosynthetic genes have been deleted or otherwise rendered inactive and in which at least one megalomicin biosynthetic gene has been introduced.

For example, the present invention enables one to express the megosamine genes in a *Saccharopolyspora erythraea eryG* mutant in which the erythromycin C made by this mutant is converted to megalomicin A. Alternatively, one could use an erythromycin C high -producing strain of *S. erythraea* in biotransformation methods in which the erythromycin C is fed to a *Streptomyces lividans* strain carrying only the megosamine biosynthesis and glycosyltransferase genes. As another alternative, one could use a strain of *S. lividans* that carries suitable erythromycin production genes along with the daunosamine biosynthesis genes plus *geneX* and *geneY* of Figure 5, or all of the megosamine biosynthesis genes, to produce megalomicin A.

All or some of the megalomicin gene cluster can be easily cloned under control of a suitable promoter in pCK7 or pSET152 either in one or two plasmids and introduced into the *Saccharopolyspora erythraea eryG* mutant. The *actII-ORF4/actI*p system and the phiC31/*int* system in pSET function well in this

organism (see Rowe *et al.*, 1998, *Gene*, 216:215-23, incorporated herein by reference). Alternatively, the megosamine biosynthesis genes are introduced into *Streptomyces lividans* on the same plasmids and the production of megalomicin A or its precursor mediated by bioconversion, done by feeding erythronolide B, 3-alpha-mycarosylerythronolide B, erythromycin D or erythromycin C to the *S. lividans* strain.

Lack of adequate resistance to megalomicin A in *S. erythraea* or *S. lividans* is not expected, because both organisms have MLS resistance genes (*ermE* and *mgt/lrm*, respectively), which confer resistance to several 14-membered macrolides (see Cundliffe, 1989, *Annu. Rev. Microbiol. 43*:207-33; Jenkins and Cundliffe, 1991, *Gene 108*:55-62; and Cundliffe, 1992, *Gene*, *115*:75-84, each of which is incorporated herein by reference). One can also readily determine the level of resistance of the *S. erythraea eryG* mutant and the *S. lividans* host cells to megalomicin A, both in plate tests and in liquid medium. One can repeat the bioconversion method using an *eryG* mutant of a high erythromycin A producing *S. erythraea* strain (or an *eryB* or *eryC* mutant, as necessary) to determine the level at which megalomicin A can be produced. Furthermore, if experience shows that high level megalomicin A production requires a higher level of resistance to this macrolide than present in *S. erythraea* or *S. lividans*, the necessary megalomicin self-resistance genes will be cloned from *M. megalomicea* and moved into either one of the heterologous hosts. This will be straightforward work since self-resistance genes are usually found in the cluster of macrolide biosynthesis genes and can be identified by their homology to known macrolide resistance genes and(or) by the resistance phenotype they impart to a strain that normally is sensitive.

Alternatively, *geneX* and *geneY* (Figure 5) can be added to cassettes containing the relevant daunosamine (*dnm*) biosynthesis genes (Figure 5) to provide the ability to make TDP-megosamine *in vivo* and attach it to an erythromycin algycone. The TDP-daunosamine biosynthesis genes can be re-cloned from *Streptomyces peucetius* on two compatible and mutually selectable plasmids. When an *S. lividans* strain containing these two plasmids and the *dnmS* gene for TDP-daunosamine glycosyltransferase is grown in the presence of added epsilon-rhodomycinone, its glycoside with L-daunosamine, called rhodomycin D,

is produced in good yield. Thus, bioconversion of one of the erythromycins to megalomicin A should be observed when *geneX* and *geneY* are present. One can construct all five combination - the two *N*-dimethyltransferase genes and the three glycosyltransferase genes - to discriminate *geneX* and *geneY* from those connected with mycarose and desosamine biosynthesis and attachment in the megalomicin pathway.

Because the timing of megosamine addition is unknown, one can test erythronolide B, 3-alpha-mycarosylerythronolide B, erythromycin D and erythromycin C as substrates provided to a strain that expresses the megosamine biosynthetic and transferase genes. There is need to test the C3''' and(or) C4''' acylated metabolites like megalomicin C1, because these metabolites are made from megalomicin A and not the converse, based on the precedents in the biosynthesis of tylosin (see Arisawa *et al.*, 1994, *Appl. Environ. Microbiol. 60*: 2657-2661), carbomycin (see Epp *et al.*, 1989, *Gene 85*:293-301), and midecamycin (see Hara and Hutchinson, 1992, *J. Bacteriol. 174*, 5141-5144). If C-6 glycosylation of erythronolide B or 3-alpha-mycarosylerythronolide B (Figure 5) happens before addition of desosamine to C-5, then the erythromycin genes might not be able to complete formation of megalomicin A from some mono or diglycoside if the erythromycin glycosyltransferases cannot tolerate a C-6 glycoside. Although unexpected, such an outcome could be circumvented in accordance with the methods of the invention by cloning further megalomicin biosynthesis genes into the appropriate *S. erythraea* background or into *S. lividans* – specifically, the necessary deoxysugar biosynthesis and attachment genes – to create a recombinant strain that produces megalomicin A.

The acyltransferase gene that adds acetate or propionate to the C3''' or C4''' positions of mycarose in megalomicin B, C1 and C2 (Figure 3) is contained within the cosmids of the invention and can be identified by scanning the sequence data for the megalomicin gene cluster to locate homologs of *carE* and *mdmB* or their *acyA* homologs from the tylosin producer. The *carE* and *acyA* genes govern C4''' acylation in the carbomycin and tylosin pathway, respectively. The megalomicin homolog has the equivalent function in megalomicin biosynthesis (but is specific for C3''' and C4''' acylation). The gene can be cloned under control of a suitable promoter and introduced into *S. lividans* to produce the

desired acyl derivative of megalomicin A. Alternatively, introduction of the *carE* gene can form megalomicin B. This gene can be cloned from the carbomycin, spiramycin or tylosin producers.

If the amount of megalomicin produced by an *S. erythraea* or *S. lividans* or other recombinant host cell is less than desired, yield can be improved by optimizing the growth medium and fermentation conditions, by increasing expression of the gene(s) that appear to be rate limiting, based on the level of pathway intermediates that are accumulated by the recombinant strain constructed, and by reconstructing the *ery*, *dnm*, and megalomicin biosynthesis genes on vectors like pSET152 that can be integrated into the genome to provide a stabler recombinant strain for strain improvement.

In another embodiment, the present invention provides recombinant vectors encoding one or more of the megosamine, desosamine, and mycarose biosynthetic and transfer genes and heterologous host cells comprising those vectors. In this embodiment of the invention, the heterologous host cell is typically a cell that is unable to produce the sugar and transfer it to a polyketide unless the vector of the invention is introduced. For example, neither *Streptomyces lividans* nor *S. coelicolor* is naturally capable of making megosamine, desosamine, or mycarose or transferring those moieties to a polyketide. However, the present invention provides recombinant *Streptomyces lividans* and *S. coelicolor* host cells that are capable of making megosamine, desosamine, and/or mycarose and transferring those moieties to a polyketide.

Moreover, additional recombinant gene products can be expressed in the host cell to improve production of a desired polyketide. As but one non-limiting example, certain of the recombinant PKS proteins of the invention may produce a polyketide other than or in addition to the predicted polyketide, because the polyketide is cleaved from the PKS by the thioesterase (TE) domain in module 6 prior to processing by other domains on the PKS, in particular, any KR, DH, and/or ER domains in module 6. The production of the predicted polyketide can be increased in such instances by deleting the TE domain coding sequences from the gene and, optionally, expressing the TE domain as a separate protein. See Gokhale *et al.*, Feb. 1999, "Mechanism and specificity of the terminal thioesterase

domain from the erythromycin polyketide synthase," *Chem. & Biol. 6*: 117-125, incorporated herein by reference.

Thus, in one important aspect, the present invention provides methods, expression vectors, and recombinant host cells that enable the production of megalomicin and hydroxylated and glycosylated derivatives of megalomicin in heterologous host cells. The present invention also provides methods for making a wide variety of polyketides derived in part from the megalomicin PKS or other biosynthetic genes, as described in the following Section.

Section VI: Hybrid PKS Genes

The present invention provides recombinant DNA compounds encoding each of the domains of each of the modules of the megalomicin PKS as well as the other megalomicin biosynthetic enzymes. The availability of these compounds permits their use in recombinant procedures for production of desired portions of the megalomicin PKS fused to or expressed in conjunction with all or a portion of a heterologous PKS and, optionally, one or more polyketide modification enzymes. These compounds also permit the modification of polyketides with the various megalomicin modification enzymes. The resulting hybrid PKS can then be expressed in a host cell to produce a desired polyketide or modified form thereof.

Thus, in accordance with the methods of the invention, a portion of the megalomicin biosynthetic gene coding sequence that encodes a particular activity can be isolated and manipulated, for example, to replace the corresponding region in a different modular PKS gene or modification enzyme gene. In addition, coding sequences for individual proteins, modules, domains, and portions thereof of the megalomicin PKS can be ligated into suitable expression systems and used to produce the portion of the protein encoded. The resulting protein can be isolated and purified or can may be employed *in situ* to effect polyketide synthesis. Depending on the host for the recombinant production of the domain, module, protein, or combination of proteins, suitable control sequences such as promoters, termination sequences, enhancers, and the like are ligated to the nucleotide sequence encoding the desired protein in the construction of the expression vector, as described above.

In one important embodiment, the invention thus provides hybrid PKS enzymes and the corresponding recombinant DNA compounds that encode those hybrid PKS enzymes. For purposes of the invention, a hybrid PKS is a recombinant PKS that comprises all or part of one or more extender modules, loading module, and/or thioesterase/cyclase domain of a first PKS and all or part of one or more extender modules, loading module, and/or thioesterase/cyclase domain of a second PKS. In one preferred embodiment, the first PKS is most but not all of the megalomicin PKS, and the second PKS is only a portion of a non-megalomicin PKS. An illustrative example of such a hybrid PKS includes a megalomicin PKS in which the megalomicin PKS loading module has been replaced with a loading module of another PKS. Another example of such a hybrid PKS is a megalomicin PKS in which the AT domain of extender module 3 is replaced with an AT domain that binds only malonyl CoA. In another preferred embodiment, the first PKS is most but not all of a non-megalomicin PKS, and the second PKS is only a portion of the megalomicin PKS. An illustrative example of such a hybrid PKS includes a rapamycin PKS in which an AT specific for malonyl CoA is replaced with the AT from the megalomicin PKS specific for methylmalonyl CoA. Other illustrative hybrid PKSs of the invention are described below.

Those of skill in the art will recognize that all or part of either the first or second PKS in a hybrid PKS of the invention need not be isolated from a naturally occurring source. For example, only a small portion of an AT domain determines its specificity. See PCT patent application No. WO US99/15047, and Lau *et al.*, *infra,* incorporated herein by reference. The state of the art in DNA synthesis allows the artisan to construct *de novo* DNA compounds of size sufficient to construct a useful portion of a PKS module or domain. Thus, the desired derivative coding sequences can be synthesized using standard solid phase synthesis methods such as those described by Jaye *et al.*, 1984, *J. Biol. Chem. 259*: 6331, and instruments for automated synthesis are available commercially from, for example, Applied Biosystems, Inc. For purposes of the invention, such synthetic DNA compounds are deemed to be a portion of a PKS.

With this general background regarding hybrid PKSs of the invention, one can better appreciate the benefit provided by the DNA compounds of the invention

that encode the individual domains, modules, and proteins that comprise the megalomicin PKS. As described above, the megalomicin PKS is comprised of a loading module, six extender modules composed of a KS, AT, ACP, and zero, one, two, or three KR, DH, and ER domains, and a thioesterase domain. The DNA compounds of the invention that encode these domains individually or in combination are useful in the construction of the hybrid PKS encoding DNA compounds of the invention. For example, a DNA compound of the invention that encodes an extender module or portion of an extender module is useful in the construction of a coding sequence that encodes a protein subcomponent of a PKS. The DNA compound of the invention that comprises a coding sequence of a PKS subunit protein is useful in the construction of an expression vector that drives expression of the subunit in a host cell that expresses the other subunits and so produces a functional PKS.

The recombinant DNA compounds of the invention that encode the loading module of the megalomicin PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the megalomicin PKS loading module is inserted into a DNA compound that comprises the coding sequence for one or more heterologous PKS extender modules. The resulting construct, in which the coding sequence for the loading module of the heterologous PKS is replaced by that for the coding sequence of the megalomicin PKS loading module provides a novel PKS. Examples include the DEBS, rapamycin, FK-506, FK-520, rifamycin, and avermectin PKS coding sequences. In another embodiment, a DNA compound comprising a sequence that encodes the megalomicin PKS loading module is inserted into a DNA compound that comprises the coding sequence for the megalomicin PKS or a recombinant megalomicin PKS that produces a megalomicin derivative.

In another embodiment, a portion of the loading module coding sequence is utilized in conjuction with a heterologous coding sequence. In this embodiment, the invention provides, for example, replacing the methylmalonyl CoA (propionyl) specific AT with a malonyl CoA (acetyl), ethylmalonyl CoA (butyryl), or other CoA specific AT. In addition, the AT and/or ACP can be replaced by another AT and/or another ACP or an inactivated KS, such as a KS^Q, an AT, and/or another

ACP. The resulting heterologous loading module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes megalomicin, a megalomicin derivative, or another polyketide.

The recombinant DNA compounds of the invention that encode the first extender module of the megalomicin PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the megalomicin PKS first extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the first extender module of the megalomicin PKS or the latter is merely added to coding sequences for modules of the heterologous PKS, provides a novel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encodes the first extender module of the megalomicin PKS is inserted into a DNA compound that comprises coding sequences for the megalomicin PKS or a recombinant megalomicin PKS that produces a megalomicin derivative.

In another embodiment, a portion or all of the first extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting (which includes inactivating) the KR; inserting a DH or a DH and ER; and/or replacing the KR with another KR, a DH and KR, or a DH, KR, and ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the megalomicin PKS, from a gene for a PKS that produces a polyketide other than megalomicin, or from chemical synthesis. The resulting heterologous first extender module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes megalomicin, a megalomicin derivative, or another polyketide.

Those of skill in the art will recognize, however, that deletion of the KR domain of extender module 1 or insertion of a DH domain or DH and KR domains

into extender module 1 will prevent the typical cyclization of the polyketide at the hydroxyl group created by the KR if such hybrid module is employed as a first extender module in a hybrid PKS or is otherwise involved in producing a portion of the polyketide at which cyclization is to occur. Such deletions or insertions can be useful, however, to create linear molecules or to induce cyclization at another site in the molecule.

As noted above, the invention also provides recombinant PKSs and recombinant DNA compounds and vectors that encode such PKSs in which the KS domain of the first extender module has been inactivated. Such constructs are typically expressed in translational reading frame with the first two extender modules on a single protein, with the remaining modules and domains of a megalomicin, megalomicin derivative, or hybrid PKS expressed as one or more, typically two, proteins to form the multi-protein functional PKS. The utility of these constructs is that host cells expressing, or cell free extracts containing, the PKS encoded thereby can be fed or supplied with N-acylcysteamine thioesters of precursor molecules to prepare megalomicin derivative compounds. See U.S. patent application Serial No. 09/492,733, filed 27 Jan. 2000, and PCT publication Nos. WO 00/44717, 99/03986 and 97/02358, each of which is incorporated herein by reference.

The recombinant DNA compounds of the invention that encode the second extender module of the megalomicin PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the megalomicin PKS second extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the second extender module of the megalomicin PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS. In another embodiment, a DNA compound comprising a sequence that encodes the second extender module of the megalomicin PKS is inserted into a DNA compound that comprises the coding sequences for the megalomicin PKS or a recombinant megalomicin PKS that produces a megalomicin derivative.

In another embodiment, a portion or all of the second extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting (or inactivating) the KR; replacing the KR with a KR, a KR and a DH, or a KR, DH, and ER; and/or inserting a DH or a DH and an ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the megalomicin PKS, from a coding sequence for a PKS that produces a polyketide other than megalomicin, or from chemical synthesis. The resulting heterologous second extender module coding sequence can be utilized in conjunction with a coding sequence from a PKS that synthesizes megalomicin, a megalomicin derivative, or another polyketide.

The recombinant DNA compounds of the invention that encode the third extender module of the megalomicin PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the megalomicin PKS third extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the third extender module of the megalomicin PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS. In another embodiment, a DNA compound comprising a sequence that encodes the third extender module of the megalomicin PKS is inserted into a DNA compound that comprises coding sequences for the megalomicin PKS or a recombinant megalomicin PKS that produces a megalomicin derivative.

In another embodiment, a portion or all of the third extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting the inactive KR; and/or

replacing the KR with an active KR, or a KR and DH, or a KR, DH, and ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the megalomicin PKS, from a gene for a PKS that produces a polyketide other than megalomicin, or from chemical synthesis. The resulting heterologous third extender module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes megalomicin, a megalomicin derivative, or another polyketide.

The recombinant DNA compounds of the invention that encode the fourth extender module of the megalomicin PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the megalomicin PKS fourth extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the fourth extender module of the megalomicin PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS. In another embodiment, a DNA compound comprising a sequence that encodes the fourth extender module of the megalomicin PKS is inserted into a DNA compound that comprises coding sequences for the megalomicin PKS or a recombinant megalomicin PKS that produces a megalomicin derivative.

In another embodiment, a portion of the fourth extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting or inactivating any one, two, or all three of the ER, DH, and KR; and/or replacing any one, two, or all three of the ER, DH, and KR with either a KR, a DH and KR, or a KR, DH, and ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the megalomicin PKS (except for the DH and ER domains), from a coding sequence

for a PKS that produces a polyketide other than megalomicin, or from chemical synthesis. The resulting heterologous fourth extender module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes megalomicin, a megalomicin derivative, or another polyketide.

The recombinant DNA compounds of the invention that encode the fifth extender module of the megalomicin PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the megalomicin PKS fifth extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the fifth extender module of the megalomicin PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS. In another embodiment, a DNA compound comprising a sequence that encodes the fifth extender module of the megalomicin PKS is inserted into a DNA compound that comprises the coding sequence for the megalomicin PKS or a recombinant megalomicin PKS that produces a megalomicin derivative.

In another embodiment, a portion or all of the fifth extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting (or inactivating) the KR; inserting a DH or a DH and ER; and/or replacing the KR with another KR, a DH and KR, or a DH, KR, and ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the megalomicin PKS, from a coding sequence for a PKS that produces a polyketide other than megalomicin, or from chemical synthesis. The resulting heterologous fifth extender module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes megalomicin, a megalomicin derivative, or another polyketide.

The recombinant DNA compounds of the invention that encode the sixth extender module of the megalomicin PKS and the corresponding polypeptides

encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the megalomicin PKS sixth extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the sixth extender module of the megalomicin PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS. In another embodiment, a DNA compound comprising a sequence that encodes the sixth extender module of the megalomicin PKS is inserted into a DNA compound that comprises the coding sequences for the megalomicin PKS or a recombinant megalomicin PKS that produces a megalomicin derivative.

In another embodiment, a portion or all of the sixth extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting or inactivating the KR or replacing the KR with another KR, a KR and DH, or a KR, DH, and an ER; and/or inserting a DH or a DH and ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the megalomicin PKS, from a coding sequence for a PKS that produces a polyketide other than megalomicin, or from chemical synthesis. The resulting heterologous sixth extender module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes megalomicin, a megalomicin derivative, or another polyketide.

The sixth extender module of the megalomicin PKS is followed by a thioesterase domain. This domain is important in the cyclization of the polyketide and its cleavage from the PKS. The present invention provides recombinant DNA compounds that encode hybrid PKS enzymes in which the megalomicin PKS is fused to a heterologous thioesterase or a heterologous PKS is fused to the megalomicin PKS thioesterase. Thus, for example, a thioesterase domain coding sequence from another PKS gene can be inserted at the end of the sixth (or other final) extender module coding sequence in recombinant DNA compounds of the

invention or the megalomicin PKS thioesterase can be similarly fused to a heterologous PKS. Recombinant DNA compounds encoding this thioesterase domain are useful in constructing DNA compounds that encode the megalomicin PKS, a PKS that produces a megalomicin derivative, and a PKS that produces a polyketide other than megalomicin or a megalomicin derivative.

Thus, the hybrid modules of the invention are incorporated into a PKS to provide a hybrid PKS of the invention. A hybrid PKS of the invention can result not only:

(i) from fusions of heterologous domain (where heterologous means the domains in a module are derived from at least two different naturally occurring modules) coding sequences to produce a hybrid module coding sequence contained in a PKS gene whose product is incorporated into a PKS,

but also:

(ii) from fusions of heterologous modules (where heterologous module means two modules are adjacent to one another that are not adjacent to one another in naturally occurring PKS enzymes) coding sequences to produce a hybrid coding sequence contained in a PKS gene whose product is incorporated into a PKS,

(iii) from expression of one or more megalomicin PKS genes with one or more non-megalomicin PKS genes, including both naturally occurring and recombinant non-megalomicin PKS genes, and

(iv) from combinations of the foregoing.

Various hybrid PKSs of the invention illustrating these various alternatives are described herein.

An example of a hybrid PKS comprising fused modules results from fusion of the loading module of either the DEBS PKS or the narbonolide PKS (see PCT patent application No. US99/11814, incorporated herein by reference) with extender modules 1 and 2 of the megalomicin PKS to produce a hybrid *megAI* gene. Co-expression of either one of these two hybrid *megAI* genes with the *megAII* and meg*AIII* genes in suitable host cells, such as *Streptomcyes lividans*, results in expression of a hybrid PKS of the invention that produces 6-deoxyerythronolide B (the polyketide product of the natural *megA* genes) in recombinant host cells. Co-expression of either one of these two hybrid *megAI*

genes with the *eryAII* and *eryAIII* genes similarly results in the production of 6-dEB, while co-expression with the analogous narbonolide PKS genes, *picAII*, *picAIII* and *picAIV*, results in the production of 3-deoxy-3-oxo-6-dEB (3-keto-6-dEB), useful in the production of ketolides, compounds with potent anti-bacterial activity.

Another example of a hybrid PKS comprising a hybrid module is prepared by co-expressing the *megAI* and *megAII* genes with a *megAIII* hybrid gene encoding extender module 5 and the KS and AT of extender module 6 of the megalomicin PKS fused to the ACP of module 6 and the TE of the narbonolide PKS. The resulting hybrid PKS of the invention produces 3-keto-6-dEB. This compound can also be prepared by a recombinant megalomicin derivative PKS of the invention in which the KR domain of module 6 of the megalomicin PKS has been deleted. Moreover, the invention provides hybrid PKSs in which not only the above changes have been made but also the AT domain of module 6 has been replaced with a malonyl-specific AT. These hybrid PKSs produce 2-desmethyl-3-deoxy-3-oxo-6-dEB, a useful intermediate in the preparation of 2-desmethyl ketolides, compounds with potent antibiotic activity.

Another illustrative example of a hybrid PKS includes the hybrid PKS of the invention resulting only from the latter change in the hybrid PKS just described. Thus, co-expression of the *megAI* and *megAII* genes with a hybrid *megAIII* gene in which the AT domain of module 6 has been replaced by a malonyl-specific AT results in the expression of a hybrid PKS that produces 2-desmethyl-6-dEB in recombinant host cells. This compound is a useful intermediate for making 2-desmethyl erythromycins in recombinant host cells of the invention, as well as for making 2-desmethyl semi-synthetic ketolides.

While many of the hybrid PKSs described above are composed primarily of megalomicin PKS proteins, those of skill in the art recognize that the present invention provides many different hybrid PKSs, including those composed of only a small portion of the megalomicin PKS. For example, the present invention provides a hybrid PKS in which a hybrid *eryAI* gene that encodes the megalomicin PKS loading module fused to extender modules 1 and 2 of DEBS is coexpressed with the *eryAII* and *eryAIII* genes. The resulting hybrid PKS produces 6-dEB, the product of the native DEBS. When the construct is expressed in

Saccharopolyspora erythraea host cells (either via chromosomal integration in the chromosome or via a vector that encodes the hybrid PKS), the resulting recombinant host cell of the invention produces erythromycins. Another illustrative example is the hybrid PKS of the invention composed of the *megAI* and *eryAII* and *eryAIII* gene products. This construct is also useful in expressing erythromycins in *Saccharopolyspora erythraea* host cells. In a preferred embodiment, the *S. erythraea* host cells are *eryAI* mutants that do not produce 6-deoxyerythronolide B.

Another example is the hybrid PKS of the invention composed of the products of the *picAI* and *picAII* genes (the two proteins that comprise the loading module and extender modules 1 - 4, inclusive, of the narbonolide PKS) and the *megAIII* gene. The resulting hybrid PKS produces the macrolide aglycone 3-hydroxy-narbonolide in *Streptomyces lividans* host cells and the corresponding erythromycins in *Saccharopolyspora erythraea* host cells.

Each of the foregoing hybrid PKS enzymes of the invention, and the hybrid PKS enzymes of the invention generally, can be expressed in a host cell that also expresses a functional *oleP* gene product. The *oleP* gene encodes an oleandomycin modification enzyme, and expression of the gene together with a hybrid PKS of the invention provides the compounds of the invention in which a C-8 hydroxyl, a C-8a or C-8-C-8a epoxide is present.

Recombinant methods for manipulating modular PKS genes to make hybrid PKS enzymes are described in U.S. Patent Nos. 5,672,491; 5,843,718; 5,830,750; and 5,712,146; and in PCT publication Nos. 98/49315 and 97/02358, each of which is incorporated herein by reference. A number of genetic engineering strategies have been used with DEBS to demonstrate that the structures of polyketides can be manipulated to produce novel natural products, primarily analogs of the erythromycins (see the patent publications referenced *supra* and Hutchinson, 1998, *Curr Opin Microbiol. 1*:319-329, and Baltz, 1998, *Trends Microbiol. 6*:76-83, incorporated herein by reference). Because of the similar activity of the megalomicin PKS and DEBS (both PKS enzymes produce the macrolide aglycone 6-dEB), these methods can be readily applied to the recombinant megalomicin PKS genes of the invention.

These techniques include: (i) deletion or insertion of modules to control chain length, (ii) inactivation of reduction/dehydration domains to bypass beta-carbon processing steps, (iii) substitution of AT domains to alter starter and extender units, (iv) addition of reduction/dehydration domains to introduce catalytic activities, and (v) substitution of ketoreductase KR domains to control hydroxyl stereochemistry. In addition, engineered blocked mutants of DEBS have been used for precursor directed biosynthesis of analogs that incorporate synthetically derived starter units. For example, more than 100 novel polyketides were produced by engineering single and combinatorial changes in multiple modules of DEBS. Hybrid PKS enzymes based on DEBS with up to three catalytic domain substitutions were constructed by cassette mutagenesis, in which various DEBS domains were replaced with domains from the rapamycin PKS (see Schweke *et al.*, 1995, *Proc. Nat. Acad. Sci. USA 92*, 7839-7843, incorporated herein by reference) or one more of the DEBS KR domains was deleted. Functional single domain replacements or deletions were combined to generate DEBS enzymes with double and triple catalytic domain substitutions (see McDaniel *et al.*, 1999, *Proc. Nat. Acad. Sci. USA 96*, 1846-1851, incorporated herein by reference). By providing the analogous megalomicin/rapamycin hybrid PKS enzymes, the present invention provides alternative means to make these polyketides.

Methods for generating libraries of polyketides have been greatly improved by cloning PKS genes as a set of three or more mutually selectable plasmids, each carrying a different wild-type or mutant PKS gene, then introducing all possible combinations of the plasmids with wild-type, mutant, and hybrid PKS coding sequences into the same host (see U.S. patent application Serial No. 60/129,731, filed 16 Apr. 1999, and PCT Pub. No. 98/27203, each of which is incorporated herein by reference). This method can also incorporate the use of a KS1° mutant, which by mutational biosynthesis can produce polyketides made from diketide starter units (see Jacobsen *et al.*, 1997, *Science* 277, 367-369, incorporated herein by reference), as well as the use of a truncated gene that leads to 12-membered macrolides or an elongated gene that leads to 16-membered ketolides. Moreover, by utilizing in addition one or more vectors that encode glycosyl biosynthesis and transfer genes, such as those of the present invention for megosamine,

desosamine, oleandrose, cladinose, and/or mycarose (in any combination), a large collection of glycosylated polyketides can be prepared.

The following Table lists references describing illustrative PKS genes and corresponding enzymes that can be utilized in the construction of the recombinant hybrid PKSs and the corresponding DNA compounds that encode them of the invention. Also presented are various references describing tailoring enzymes and corresponding genes that can be employed in accordance with the methods of the invention.

Avermectin

U.S. Pat. No. 5,252,474 to Merck.

MacNeil *et al.*, 1993, Industrial Microorganisms: Basic and Applied Molecular Genetics, Baltz, Hegeman, & Skatrud, eds. (ASM), pp. 245-256, A Comparison of the Genes Encoding the Polyketide Synthases for Avermectin, Erythromycin, and Nemadectin.

MacNeil *et al.*, 1992, *Gene 115*: 119-125, Complex Organization of the *Streptomyces avermitilis* genes encoding the avermectin polyketide synthase.

Candicidin (FR008)

Hu *et al.*, 1994, *Mol. Microbiol.* 14: 163-172.

Epothilone

PCT Pub. No. 00/031247 to Kosan.

Erythromycin

PCT Pub. No. 93/13663 to Abbott.

US Pat. No. 5,824,513 to Abbott.

Donadio *et al.*, 1991, *Science 252*:675-9.

Cortes *et al.*, 8 Nov. 1990, *Nature 348*:176-8, An unusually large multifunctional polypeptide in the erythromycin producing polyketide synthase of *Saccharopolyspora erythraea*.

Glycosylation Enzymes

PCT Pub. No. 97/23630 to Abbott.

FK-506

Motamedi *et al.*, 1998, The biosynthetic gene cluster for the macrolactone ring of the immunosuppressant FK506, *Eur. J. biochem. 256*: 528-534.

Motamedi *et al.*, 1997, Structural organization of a multifunctional polyketide synthase involved in the biosynthesis of the macrolide immunosuppressant FK506, *Eur. J. Biochem. 244*: 74-80.

Methyltransferase

US 5,264,355, issued 23 Nov. 1993, Methylating enzyme from *Streptomyces* MA6858. 31-O-desmethyl-FK506 methyltransferase.

Motamedi *et al.*, 1996, Characterization of methyltransferase and hydroxylase genes involved in the biosynthesis of the immunosuppressants FK506 and FK520, *J. Bacteriol. 178*: 5243-5248.

FK-520

PCT Pub. No. 00/20601 to Kosan.

See also Nielsen *et al.*, 1991, *Biochem. 30*:5789-96 (enzymology of pipecolate incorporation).

Lovastatin

U.S. Pat. No. 5,744,350 to Merck.

Narbomycin (and Picromycin)

PCT Pub. No. WO US99/61599 to Kosan.

Nemadectin

MacNeil *et al.*, 1993, *supra.*

Niddamycin

Kakavas *et al.*, 1997, Identification and characterization of the niddamycin polyketide synthase genes from *Streptomyces caelestis*, *J. Bacteriol. 179*: 7515-7522.

Oleandomycin

Swan *et al.*, 1994, Characterization of a *Streptomyces antibioticus* gene encoding a type I polyketide synthase which has an unusual coding sequence, *Mol. Gen. Genet. 242*: 358-362.

PCT Pub. No. 00/026349 to Kosan.

Olano *et al.*, 1998, Analysis of a *Streptomyces antibioticus* chromosomal region involved in oleandomycin biosynthesis, which encodes two glycosyltransferases responsible for glycosylation of the macrolactone ring, *Mol. Gen. Genet. 259*(3): 299-308.

Platenolide

EP Pub. No. 791,656 to Lilly.

Rapamycin

Schwecke *et al.*, Aug. 1995, The biosynthetic gene cluster for the polyketide rapamycin, *Proc. Natl. Acad. Sci. USA 92*:7839-7843.

Aparicio *et al.*, 1996, Organization of the biosynthetic gene cluster for rapamycin in *Streptomyces hygroscopicus*: analysis of the enzymatic domains in the modular polyketide synthase, *Gene 169*: 9-16.

Rifamycin

August *et al.*, 13 Feb. 1998, Biosynthesis of the ansamycin antibiotic rifamycin: deductions from the molecular analysis of the *rif* biosynthetic gene cluster of *Amycolatopsis mediterranei* S669, *Chemistry & Biology, 5*(2): 69-79.

Soraphen

U.S. Pat. No. 5,716,849 to Novartis.

Schupp *et al.*, 1995, *J. Bacteriology 177*: 3673-3679. A *Sorangium cellulosum* (Myxobacterium) Gene Cluster for the Biosynthesis of the Macrolide Antibiotic Soraphen A: Cloning, Characterization, and Homology to Polyketide Synthase Genes from Actinomycetes.

Spiramycin

U.S. Pat. No. 5,098,837 to Lilly.

<u>Activator Gene</u>

U.S. Pat. No. 5,514,544 to Lilly.

Tylosin

EP Pub. No. 791,655 to Lilly.

Kuhstoss *et al.*, 1996, *Gene 183*:231-6., Production of a novel polyketide through the construction of a hybrid polyketide synthase.

U.S. Pat. No. 5,876,991 to Lilly.

<u>Tailoring enzymes</u>

Merson-Davies and Cundliffe, 1994, *Mol. Microbiol. 13*: 349-355. Analysis of five tylosin biosynthetic genes from the *tylBA* region of the *Streptomyces fradiae* genome.

As the above Table illustrates, there are a wide variety of PKS genes that serve as readily available sources of DNA and sequence information for use in constructing the hybrid PKS-encoding DNA compounds of the invention.

In constructing hybrid PKSs of the invention, certain general methods may be helpful. For example, it is often beneficial to retain the framework of the module to be altered to make the hybrid PKS. Thus, if one desires to add DH and ER functionalities to a module, it is often preferred to replace the KR domain of the original module with a cognate KR, DH, and ER domain-containing segment from another module, instead of merely inserting DH and ER domains. One can alter the stereochemical specificity of a module by replacement of the KS domain with a KS domain from a module that specifies a different stereochemistry. See Lau *et al.*, 1999, "Dissecting the role of acyltransferase domains of modular polyketide synthases in the choice and stereochemical fate of extender units" *Biochemistry 38*(5):1643-1651, incorporated herein by reference. One can alter the specificity of an AT domain by changing only a small segment of the domain. See Lau *et al., supra*. One can also take advantage of known linker regions in PKS proteins to link modules from two different PKSs to create a hybrid PKS. See Gokhale *et al.*, 16 Apr. 1999, Dissecting and Exploiting Intermodular Communication in Polyketide Synthases", *Science 284*: 482-485, incorporated herein by reference.

The hybrid PKS-encoding DNA compounds of the invention can be and often are hybrids of more than two PKS genes. Even where only two genes are used, there are often two or more modules in the hybrid gene in which all or part of the module is derived from a second (or third) PKS gene. Thus, as one illustrative example, the invention provides a hybrid PKS that contains the naturally occurring loading module and thioesterase domain as well as extender modules one, two, four, and six of the megalomicin PKS and further contains hybrid or heterologous extender modules three and five. Hybrid or heterologous extender modules three and five contain AT domains specific for malonyl CoA and derived from, for example, the rapamycin PKS genes.

The invention also provides libraries of PKS genes, PKS proteins, and ultimately, of polyketides, that are constructed by generating modifications in the megalomicin PKS so that the protein complexes produced have altered activities in one or more respects and thus produce polyketides other than the natural product of the PKS. Novel polyketides may thus be prepared, or polyketides in general prepared more readily, using this method. By providing a large number of

different genes or gene clusters derived from a naturally occurring PKS gene cluster, each of which has been modified in a different way from the native cluster, an effectively combinatorial library of polyketides can be produced as a result of the multiple variations in these activities. As will be further described below, the metes and bounds of this embodiment of the invention can be described on the polyketide, protein, and the encoding nucleotide sequence levels.

As described above, a modular PKS "derived from" the megalomicin or other naturally occurring PKS includes a modular PKS (or its corresponding encoding gene(s)) that retains the scaffolding of the utilized portion of the naturally occurring gene. Not all modules need be included in the constructs; however, the constructs can also comprise more than six modules. On the constant scaffold, at least one enzymatic activity is mutated, deleted, replaced, or inserted so as to alter the activity of the resulting PKS relative to the original (native) PKS. Alteration results when these activities are deleted or are replaced by a different version of the activity, or simply mutated in such a way that a polyketide other than the natural product results from these collective activities. This occurs because there has been a resulting alteration of the starter unit and/or extender unit, stereochemistry, chain length or cyclization, and/or reductive or dehydration cycle outcome at a corresponding position in the product polyketide. Where a deleted activity is replaced, the origin of the replacement activity may come from a corresponding activity in a different naturally occurring PKS or from a different region of the megalomicin PKS. Any or all of the megalomicin PKS genes may be included in the derivative or portions of any of these may be included, but the scaffolding of a functional PKS protein is retained in whatever derivative is constructed. The derivative preferably contains a thioesterase activity from the megalomicin or another PKS.

Thus, a PKS derived from the megalomicin PKS includes a PKS that contains the scaffolding of all or a portion of the megalomicin PKS. The derived PKS also contains at least two extender modules that are functional, preferably three extender modules, and more preferably four or more extender modules, and most preferably six extender modules. The derived PKS also contains mutations, deletions, insertions, or replacements of one or more of the activities of the functional modules of the megalomicin PKS so that the nature of the resulting

polyketide is altered at both the protein and DNA sequence levels. Particular preferred embodiments include those wherein a KS, AT, or ACP domain has been deleted or replaced by a version of the activity from a different PKS or from another location within the same PKS. Also preferred are derivatives where at least one non-condensation cycle enzymatic activity (KR, DH, or ER) has been deleted or added or wherein any of these activities has been mutated so as to change the structure of the polyketide synthesized by the PKS.

Conversely, also included within the definition of a PKS derived from the megalomicin PKS are functional non-megalomicin PKS modules or their encoding genes wherein at least one domain or coding sequence therefor of a megalomicin PKS module has been inserted. Exemplary is the use of the megalomicin AT for extender module 2, which accepts a methylmalonyl CoA extender unit rather than malonyl CoA, to replace a malonyl specific AT in another PKS. Other examples include insertion of portions of non-condensation cycle enzymatic activities or other regions of megalomicin synthase activity into a heterologous PKS at both the DNA and protein levels.

Thus, there are at least five degrees of freedom for constructing a hybrid PKS in terms of the polyketide that will be produced. First, the polyketide chain length is determined by the number of extender modules in the PKS, and the present invention includes hybrid PKSs that contain 6, as wells as fewer or more than 6, extender modules. Second, the nature of the carbon skeleton of the PKS is determined by the specificities of the acyl transferases that determine the nature of the extender units at each position, e.g., malonyl, methylmalonyl, ethylmalonyl, or other substituted malonyl. Third, the loading module specificity also has an effect on the resulting carbon skeleton of the polyketide. The loading module may use a different starter unit, such as acetyl, butyryl, and the like. As noted above, another method for varying loading module specificity involves inactivating the KS activity in extender module 1 (KS1) and providing alternative substrates, called diketides, that are chemically synthesized analogs of extender module 1 diketide products, for extender module 2. This approach was illustrated in PCT publication Nos. 97/02358 and 99/03986, incorporated herein by reference, wherein the KS1 activity was inactivated through mutation. Fourth, the oxidation state at various positions of the polyketide will be determined by the dehydratase and reductase

portions of the modules. This will determine the presence and location of ketone and alcohol moieties and C-C double bonds or C-C single bonds in the polyketide.

Finally, the stereochemistry of the resulting polyketide is a function of three aspects of the synthase. The first aspect is related to the AT/KS specificity associated with substituted malonyls as extender units, which affects stereochemistry only when the reductive cycle is missing or when it contains only a ketoreductase, as the dehydratase would abolish chirality. Second, the specificity of the ketoreductase may determine the chirality of any beta-OH. Finally, the enoylreductase specificity for substituted malonyls as extender units may influence the stereochemistry when there is a complete KR/DH/ER available.

Thus, the modular PKS systems generally and the megalomicin PKS system particularly permit a wide range of polyketides to be synthesized. As compared to the aromatic PKS systems, the modular PKS systems accept a wider range of starter units, including aliphatic monomers (acetyl, propionyl, butyryl, isovaleryl, and the like.), aromatics (aminohydroxybenzoyl), alicyclics (cyclohexanoyl), and heterocyclics (thiazolyl). Certain modular PKSs have relaxed specificity for their starter units (Kao *et al.*, 1994, *Science, supra*). Modular PKSs also exhibit considerable variety with regard to the choice of extender units in each condensation cycle. The degree of beta-ketoreduction following a condensation reaction can be altered by genetic manipulation (Donadio *et al.*, 1991, *Science, supra*; Donadio *et al.*, 1993, *Proc. Natl. Acad. Sci. USA 90*: 7119-7123). Likewise, the size of the polyketide product can be varied by designing mutants with the appropriate number of modules (Kao *et al.*, 1994, *J. Am. Chem. Soc. 116*:11612-11613). Lastly, modular PKS enzymes are particularly well known for generating an impressive range of asymmetric centers in their products in a highly controlled manner. The polyketides, antibiotics, and other compounds produced by the methods of the invention are typically single stereoisomeric forms. Although the compounds of the invention can occur as mixtures of stereoisomers, it may be beneficial in some instances to generate individual stereoisomers. Thus, the combinatorial potential within modular PKS pathways based on any naturally occurring modular, such as the megalomicin, PKS scaffold is virtually unlimited.

While hybrid PKSs are most often produced by "mixing and matching" portions of PKS coding sequences, mutations in DNA encoding a PKS can also be used to introduce, alter, or delete an activity in the encoded polypeptide. Mutations can be made to the native sequences using conventional techniques. The substrates for mutation can be an entire cluster of genes or only one or two of them; the substrate for mutation may also be portions of one or more of these genes. Techniques for mutation include preparing synthetic oligonucleotides including the mutations and inserting the mutated sequence into the gene encoding a PKS subunit using restriction endonuclease digestion. See, *e.g.*, Kunkel, 1985, *Proc. Natl. Acad. Sci. USA 82*: 448; Geisselsoder *et al.*, 1987, *BioTechniques 5*:786. Alternatively, the mutations can be effected using a mismatched primer (generally 10-20 nucleotides in length) that hybridizes to the native nucleotide sequence, at a temperature below the melting temperature of the mismatched duplex. The primer can be made specific by keeping primer length and base composition within relatively narrow limits and by keeping the mutant base centrally located. See Zoller and Smith, 1983, *Methods Enzymol. 100*:468. Primer extension is effected using DNA polymerase, the product cloned, and clones containing the mutated DNA, derived by segregation of the primer extended strand, selected. Identification can be accomplished using the mutant primer as a hybridization probe. The technique is also applicable for generating multiple point mutations. *See*, *e.g.*, Dalbie-McFarland *et al.*, 1982, *Proc. Natl. Acad. Sci. USA 79*: 6409. PCR mutagenesis can also be used to effect the desired mutations.

Random mutagenesis of selected portions of the nucleotide sequences encoding enzymatic activities can also be accomplished by several different techniques known in the art, e.g., by inserting an oligonucleotide linker randomly into a plasmid, by irradiation with X-rays or ultraviolet light, by incorporating incorrect nucleotides during *in vitro* DNA synthesis, by error-prone PCR mutagenesis, by preparing synthetic mutants, or by damaging plasmid DNA *in vitro* with chemicals, in accordance with the methods of the present invention. Chemical mutagens include, for example, sodium bisulfite, nitrous acid, nitrosoguanidine, hydroxylamine, agents which damage or remove bases thereby preventing normal base-pairing such as hydrazine or formic acid, analogues of nucleotide precursors such as 5-bromouracil, 2-aminopurine, or acridine

intercalating agents such as proflavine, acriflavine, quinacrine, and the like. Generally, plasmid DNA or DNA fragments are treated with chemical mutagens, transformed into *E. coli* and propagated as a pool or library of mutant plasmids.

In constructing a hybrid PKS of the invention, regions encoding enzymatic activity, i.e., regions encoding corresponding activities from different PKS synthases or from different locations in the same PKS, can be recovered, for example, using PCR techniques with appropriate primers. By "corresponding" activity encoding regions is meant those regions encoding the same general type of activity. For example, a KR activity encoded at one location of a gene cluster "corresponds" to a KR encoding activity in another location in the gene cluster or in a different gene cluster. Similarly, a complete reductase cycle could be considered corresponding. For example, KR/DH/ER can correspond to a KR alone.

If replacement of a particular target region in a host PKS is to be made, this replacement can be conducted *in vitro* using suitable restriction enzymes. The replacement can also be effected *in vivo* using recombinant techniques involving homologous sequences framing the replacement gene in a donor plasmid and a receptor region in a recipient plasmid. Such systems, advantageously involving plasmids of differing temperature sensitivities are described, for example, in PCT publication No. WO 96/40968, incorporated herein by reference. The vectors used to perform the various operations to replace the enzymatic activity in the host PKS genes or to support mutations in these regions of the host PKS genes can be chosen to contain control sequences operably linked to the resulting coding sequences in a manner such that expression of the coding sequences can be effected in an appropriate host.

However, simple cloning vectors may be used as well. If the cloning vectors employed to obtain PKS genes encoding derived PKS lack control sequences for expression operably linked to the encoding nucleotide sequences, the nucleotide sequences are inserted into appropriate expression vectors. This need not be done individually, but a pool of isolated encoding nucleotide sequences can be inserted into expression vectors, the resulting vectors transformed or transfected into host cells, and the resulting cells plated out into individual colonies. The invention provides a variety of recombinant DNA

compounds in which the various coding sequences for the domains and modules of the megalomicin PKS are flanked by non-naturally occurring restriction enzyme recognition sites.

The various PKS nucleotide sequences can be cloned into one or more recombinant vectors as individual cassettes, with separate control elements, or under the control of, e.g., a single promoter. The PKS subunit encoding regions can include flanking restriction sites to allow for the easy deletion and insertion of other PKS subunit encoding sequences so that hybrid PKSs can be generated. The design of such unique restriction sites is known to those of skill in the art and can be accomplished using the techniques described above, such as site-directed mutagenesis and PCR.

The expression vectors containing nucleotide sequences encoding a variety of PKS enzymes for the production of different polyketides are then transformed into the appropriate host cells to construct the library. In one straightforward approach, a mixture of such vectors is transformed into the selected host cells and the resulting cells plated into individual colonies and selected to identify successful transformants. Each individual colony has the ability to produce a particular PKS synthase and ultimately a particular polyketide. Typically, there will be duplications in some, most, or all of the colonies; the subset of the transformed colonies that contains a different PKS in each member colony can be considered the library. Alternatively, the expression vectors can be used individually to transform hosts, which transformed hosts are then assembled into a library. A variety of strategies are available to obtain a multiplicity of colonies each containing a PKS gene cluster derived from the naturally occurring host gene cluster so that each colony in the library produces a different PKS and ultimately a different polyketide. The number of different polyketides that are produced by the library is typically at least four, more typically at least ten, and preferably at least 20, and more preferably at least 50, reflecting similar numbers of different altered PKS gene clusters and PKS gene products. The number of members in the library is arbitrarily chosen; however, the degrees of freedom outlined above with respect to the variation of starter, extender units, stereochemistry, oxidation state, and chain length enables the production of quite large libraries.

Methods for introducing the recombinant vectors of the invention into suitable hosts are known to those of skill in the art and typically include the use of $CaCl_2$ or agents such as other divalent cations, lipofection, DMSO, protoplast transformation, conjugation, infection, transfection, and electroporation. The polyketide producing colonies can be identified and isolated using known techniques and the produced polyketides further characterized. The polyketides produced by these colonies can be used collectively in a panel to represent a library or may be assessed individually for activity.

The libraries of the invention can thus be considered at four levels: (1) a multiplicity of colonies each with a different PKS encoding sequence; (2) the proteins produced from the coding sequences; (3) the polyketides produced from the proteins assembled into a functional PKS; and (4) antibiotics or compounds with other desired activities derived from the polyketides. Of course, combination libraries can also be constructed wherein members of a library derived, for example, from the megalomicin PKS can be considered as a part of the same library as those derived from, for example, the rapamycin PKS or DEBS.

Colonies in the library are induced to produce the relevant synthases and thus to produce the relevant polyketides to obtain a library of polyketides. The polyketides secreted into the media can be screened for binding to desired targets, such as receptors, signaling proteins, and the like. The supernatants *per se* can be used for screening, or partial or complete purification of the polyketides can first be effected. Typically, such screening methods involve detecting the binding of each member of the library to receptor or other target ligand. Binding can be detected either directly or through a competition assay. Means to screen such libraries for binding are well known in the art and can be applied in accordance with the methods of the present invention. Alternatively, individual polyketide members of the library can be tested against a desired target. In this event, screens wherein the biological response of the target is measured can more readily be included. Antibiotic activity can be verified using typical screening assays such as those set forth in Lehrer *et al.*, 1991, *J. Immunol. Meth. 137*:167-173, incorporated herein by reference, and in the Examples below.

The invention provides methods for the preparation of a large number of polyketides. These polyketides are useful intermediates in formation of

compounds with antibiotic or other activity through hydroxylation, epoxidation, and glycosylation reactions as described above. In general, the polyketide products of the PKS must be further modified, typically by hydroxylation and glycosylation, to exhibit potent antibiotic activity. Hydroxylation results in the novel polyketides of the invention that contain hydroxyl groups at C-6, which can be accomplished using the hydroxylase encoded by the *eryF* gene, and/or C-12, which can be accomplished using the hydroxylase encoded by the *picK* or *eryK* gene. Also, the *oleP* gene is available in recombinant form, which can be used to express the *oleP* gene product in any host cell. A host cell, such as a *Streptomyces* host cell or a *Saccharopolyspora erythraea* host cell, modified to express the *oleP* gene thus can be used to produce polyketides comprising the C-8-C-8a epoxide present in oleandomycin. Thus the invention provides such modified polyketides. The presence of hydroxyl groups at these positions can enhance the antibiotic activity of the resulting compound relative to its unhydroxylated counterpart.

Methods for glycosylating polyketides are generally known in the art and can be applied in accordance with the methods of the present invention; the glycosylation may be effected intracellularly by providing the appropriate glycosylation enzymes or may be effected *in vitro* using chemical synthetic means as described herein and in PCT publication No. WO 98/49315, incorporated herein by reference. Preferably, glycosylation with desosamine, mycarose, and/or megosamine is effected in accordance with the methods of the invention in recombinant host cells provided by the invention. In general, the approaches to effecting glycosylation mirror those described above with respect to hydroxylation. The purified enzymes, isolated from native sources or recombinantly produced may be used *in vitro*. Alternatively and as noted, glycosylation may be effected intracellularly using endogenous or recombinantly produced intracellular glycosylases. In addition, synthetic chemical methods may be employed.

The antibiotic modular polyketides may contain any of a number of different sugars, although D-desosamine, or a close analog thereof, is most common. Erythromycin, picromycin, megalomicin, narbomycin, and methymycin contain desosamine. Erythromycin also contains L-cladinose (3-O-methyl mycarose). Tylosin contains mycaminose (4-hydroxy desosamine), mycarose and

6-deoxy-D-allose. 2-acetyl-1-bromodesosamine has been used as a donor to glycosylate polyketides by Masamune *et al.*, 1975, *J. Am. Chem. Soc. 97*: 3512-3513. Other, apparently more stable donors include glycosyl fluorides, thioglycosides, and trichloroacetimidates; see Woodward *et al.*, 1981, *J. Am. Chem. Soc.* 103: 3215; Martin *et al.*, 1997, *J. Am. Chem. Soc. 119*: 3193; Toshima *et al.*, 1995, *J. Am. Chem. Soc. 117*: 3717; Matsumoto *et al.*, 1988, *Tetrahedron Lett. 29*: 3575. Glycosylation can also be effected using the polyketide aglycones as starting materials and using *Saccharopolyspora erythraea* or *Streptomyces venezuelae* or other host cell to make the conversion, preferably using mutants unable to synthesize macrolides, as discussed in the preceding Section.

Thus, a wide variety of polyketides can be produced by the hybrid PKS enzymes of the invention. These polyketides are useful as antibiotics and as intermediates in the synthesis of other useful compounds, as described in the following section.

Section VII: Host Cells Containing Multiple Expression Vectors

A recombinant host cell of the invention may contain nucleic acid encoding a megalomicin PKS domain, module, or protein, or megalomicin modification enzyme at a single genetic locus, *e.g.*, on a single plasmid or at a single chromosomal locus, or at different genetic loci, *e.g.*, on separate plasmids and/or chromosomal loci. By "multiple" is meant two or more; by "vector" is meant a nucleic acid molecule which can be used to transform host systems and which contains an independent expression system containing a coding sequence under control of a promoter and optionally a selectable marker and any other suitable sequences regulating expression. Typical such vectors are plasmids, but other vectors such as phagemids, cosmids, viral vectors and the like can be used according to the nature of the host. Of course, one or more of the separate vectors may integrate into the chromosome of the host (selection may not be required for maintenance of integrated vectors).

In one embodiment, the invention provides a recombinant host cell, which comprises at least two separate autonomously replicating recombinant DNA expression vectors, each of said vectors comprises a recombinant DNA compound encoding a megalomicin PKS domain or a megalomicin modification enzyme

operably linked to a promoter. In another embodiment, the invention provides a recombinant host cell, which comprises at least one autonomously replicating recombinant DNA expression vector and at least one modified chromosome, each of said vector(s) and each of said modified chromosome comprises a recombinant DNA compound encoding a megalomicin PKS domain or a megalomicin modification enzyme operably linked to a promoter. Preferably, the autonomously replicating recombinant DNA expression vector and/or the modified chromosome further comprises distinct selectable markers.

The above multiple-vector (chromosome) expression systems can also be used for expressing heterogeneous polyketide biosynthetic enzymes, *e.g.*, for expressing *Micromonospora megalomicea* megalomicin PKS protein, module, or domain or a megalomicin modification enzyme with a PKS protein, module, or domain, or modification enzyme from other origins in the same host cells. By placing various activities on different expression vectors, a high degree of variation can be achieved in an efficient manner. A variety of hosts can be used; any suitable host cell that can maintain multiple vectors can readily be used. Preferred hosts include *Streptomyces*, yeast, *E. coli,* other actinomycetes, and plant cells, and mammalian or insect cells or other suitable recombinant hosts can also be used. Preferred among yeast strains are *Saccharomyces cerevisiae* and *Pichia pastoris*. Preferred actinomycetes include various strains of *Streptomyces*.

If one chooses to use a host cell that does not naturally produce a polyketide, then one may need to ensure that the recombinant host is modified to also contain a holo ACP synthase activity that effects pantetheinylation of the acyl carrier protein. See PCT Pub. No. WO 97/13845, incorporated herein by reference. One of the multiple vectors may be used for this purpose. This activation step is necessary for activation of the ACP. The expression system for the holo ACP synthase may be supplied on a vector separate from that carrying a PKS coding sequence or may be supplied on the same vector or may be integrated into the chromosome of the host, or may be supplied as an expression system for a fusion protein with all or a portion of a polyketide synthase (see U.S. Patent No. 6,033,883, incorporated herein by reference).

It should be noted that in some recombinant hosts, it may also be necessary to activate the polyketides produced through postsynthesis modifications when

polyketides having such modifications are desired. If this is the case for a particular host, the host will be modified, for example by transformation, to contain those enzymes necessary for effecting these modifications. Among such enzymes, for example, are glycosylation enzymes. The use of multiple vectors can facilitate the introduction of expression systems for such enzymes.

In a preferred embodiment, the multiple vector system is used to assemble rapidly and efficiently a combinatorial library of polyketides and the PKS/modification enzymes that produce them. In an illustrative embodiment, the multiple vector system comprises four different vectors, one comprising the *megAI* gene, one the *megAII* gene, one the *megAIII* gene, and one the modification enzyme(s) gene(s). Each of these vectors can be modified to make a set of vectors. For example, one set could contain all possible AT substitutions in the loading and first and second extender modules of the megAI gene product. Another set could contain expression systems for a variety of different modification enzymes. With these four vectors sets and by combining each member of each set with each member of the other three sets, a very large library of cells, vector sets, and polyketides can be rapidly and efficiently assembled.

The combinatorial potential of a modular PKS such as the megalomicin PKS (ignoring the additional potential of different modification enzyme systems) is minimally given by: $AT_L \mathrm{X} (AT_E \mathrm{X} 4)_M$ where AT_L is the number of loading acyl transferases, AT_E is the number of extender acyl transferases, and M is the number of modules in the gene cluster. The number 4 is present in the formula because this represents the number of ways a keto group can be modified by either 1) no reaction; 2) KR activity alone; 3) KR+DH activity; or 4) KR+DH+ER activity. It has been shown that expression of only the first two modules of the erythromycin PKS resulted in the production of a predicted truncated triketide product (See Kao et al., *J. Am. Chem. Soc.*, 116:11612-11613 ((1994)). A novel 12-membered macrolide similar to methymycin aglycone was produced by expression of modules 1-5 of this PKS in *S. coelicolor* (See Kao et al., *J. Am. Chem. Soc.*, 117:9105-9106 (1995)). This work shows that PKS modules are functionally independent so that lactone ring size can be controlled by the number of modules present.

In addition to controlling the number of modules, the modules can be genetically modified, for example, by the deletion of a ketoreductase domain as described by Donadio et al., *Science*, 252:675-679 (1991); and Donadio et al., *Gene*, 115:97-103 (1992). In addition, the mutation of an enoyl reductase domain was reported by Donadio, et al., *Proc. Natl. Acad. Sci.*, 90:7119-7123 (1993). These modifications also resulted in modified PKS and thus modified polyketides.

As stated above, in the present invention, the coding sequences for catalytic activities derived from the megalomicin PKS systems found in nature can be used in their native forms or modified by standard mutagenesis techniques to delete or diminish activity or to introduce an activity into a module in which it was not originally present. For example, a KR activity can be introduced into a module normally lacking that function.

In one embodiment of the invention herein, a single host cell is modified to contain a multiplicity of vectors, each vector contributing a portion of the synthesis of a megalomicin PKS and modification enzyme (if any) system. Each of the multiple vectors for production of the megalomicin PKS system typically encodes at least two modules, and at least one of the vectors integrates into the chromosome of the host. Integration can be effected using suitable phage or integrating vectors or by homologous recombination. If homologous recombination is used, the integration event may also be designed to delete endogenous PKS genes residing in the chromosome, as described in the PCT application WO 95/08548. In these embodiments, too, a selectable marker such as hygromycin or thiostrepton resistance can be included in the vector that effects integration.

As mentioned above, additional enzymes that effect post-translational modifications to the enzyme systems in the megalomicin PKS may be introduced into the host through suitable recombinant expression systems. In addition, enzymes that activate the polyketides themselves, for example, through glycosylation may be added. It may also be desirable to modify the cell to produce more of a particular substrate utilized in polyketide biosynthesis. For example, it is generally believed that malonyl CoA levels in yeast are higher than methylmalonyl CoA; if yeast is chosen as a host, it may be desirable to increase

methylmalonyl CoA levels by the addition of one or more biosynthetic enzymes therefor.

The multiple-vector expression system can also be used to make polyketides produced by the addition of synthetic starter units to a PKS that contains an inactivated ketosynthase (KS) in the first module. As noted above, this modification permits the system to incorporate a suitable diketide thioester such as 3-hydroxy-2-methyl pantonoic acid-N-acetyl cysteamine thioester, or similar thioesters of diketide analogs, as described by Jacobsen et al., *Science*, 277:367-369 (1997). The construction of PKS modules containing inactivated ketosynthase regions can be conducted by methods known in the art, such as the method described in U.S. Patent No. 6,080,555 and PCT publication Nos. WO 99/03986 and 97/02358, each of which is incorporated herein by reference, in accordance with the methods of the present invention.

The multiple-vector expression system can be used to produce polyketides in hosts that normally do not produce them, such as *E. coli* and yeast. It also provides more efficient means to provide a variety of polyketide products by supplying the elements of the introduced PKS, whether in an *E. coli* or yeast host or in other more traditionally used hosts, such as *Streptomyces*. The invention also includes libraries of polyketides prepared using the methods of the invention.

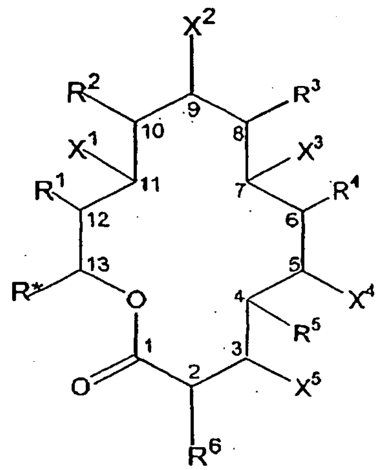
Section VIII: Compounds

The methods and recombinant DNA compounds of the invention are useful in the production of polyketides. In one important aspect, the invention provides methods for making antibiotic compounds related in structure to erythromycin, a potent antibiotic compound. The invention also provides novel ketolide compounds, polyketide compounds with potent antibiotic activity of significant interest due to activity against antibiotic resistant strains of bacteria. See Griesgraber *et al.*, 1996, *J. Antibiot. 49*: 465-477, incorporated herein by reference. Most if not all of the ketolides prepared to date are synthesized using erythromycin A, a derivative of 6-dEB, as an intermediate. In one embodiment, the present invention provides the 3-keto derivatives of the megalomicins for use as antibiotics. In particular, the 3-keto derivative of megalomicin A is a preferred ketolide of the invention. These compounds can be made chemically, substantially

in accordance with the procedures for making ketolides described in the prior art, or in recombinant host cells of the invention in which the megosamine and desosamine biosynthetic and transferase genes are present but which do not make or transfer the mycarose moiety and/or the PKS has been modified to delete the KR domain of extender module 6. The invention also provides methods for making intermediates useful in preparing traditional, 6-dEB- and erythromycin-derived ketolide compounds. See Griesgraber *et al., supra*; Agouridas *et al.*, 1998, *J. Med. Chem. 41*: 4080-4100, U.S. Patent Nos. 5,770,579; 5,760,233; 5,750,510; 5,747,467; 5,747,466; 5,656,607; 5,635,485; 5,614,614; 5,556,118; 5,543,400; 5,527,780; 5,444,051; 5,439,890; 5,439,889; and PCT publication Nos. WO 98/09978 and 98/28316, each of which is incorporated herein by reference.

As noted above, the hybrid PKS genes of the invention can be expressed in a host cell that contains the desosamine, megosamine, and/or mycarose biosynthetic genes and corresponding transferase genes as well as the required hydroxylase gene(s), which may, for example and without limitation, be either *picK*, *megK*, or *eryK* (for the C-12 position) and/or *megF* or*eryF* (for the C-6 position). The resulting compounds have antibiotic activity but can be further modified, as described in the patent publications referenced above, to yield a desired compound with improved or otherwise desired properties. Alternatively, the aglycone compounds can be produced in the recombinant host cell, and the desired glycosylation and hydroxylation steps carried out *in vitro* or *in vivo*, in the latter case by supplying the converting cell with the aglycone, as described above.

The compounds of the invention are thus optionally glycosylated forms of the polyketide set forth in formula (1) below which are hydroxylated at either the C-6 or the C-12 or both. The compounds of formula (1) can be prepared using the loading and the six extender modules of a modular PKS, modified or prepared in hybrid form as herein described. These polyketides have the formula:



(2)

including the glycosylated and isolated stereoisomeric forms thereof;

wherein R* is a straight chain, branched or cyclic, saturated or unsaturated substituted or unsubstituted hydrocarbyl of 1-15C;

each of R^1-R^6 is independently H or alkyl (1-4C) wherein any alkyl at R^1 may optionally be substituted;

each of X^1-X^5 is independently two H, H and OH, or =O; or

each of X^1-X^5 is independently H and the compound of formula (2) contains a double-bond in the ring adjacent to the position of said X at 2-3, 4-5, 6-7, 8-9 and/or 10-11;

with the proviso that:

at least two of R^1-R^6 are alkyl (1-4C).

Preferred compounds comprising formula 2 are those wherein at least three of R^1-R^5 are alkyl (1-4C), preferably methyl or ethyl; more preferably wherein at least four of R^1-R^5 are alkyl (1-4C), preferably methyl or ethyl. Also preferred are those wherein X^2 is two H, =O, or H and OH, and/or X^3 is H, and/or X^1 is OH and/or X^4 is OH and/or X^5 is OH. Also preferred are compounds with variable R* when R^1-R^5 is methyl, X^2 is =O, and X^1, X^4 and X^5 are OH. The glycosylated forms (i.e., mycarose or cladinose at C-3, desosamine at C-5, and/or megosamine at C-6) of the foregoing are also preferred.

As described above, there are a wide variety of diverse organisms that can modify compounds such as those described herein to provide compounds with or that can be readily modified to have useful activities. For example, *Saccharopolyspora erythraea* can convert 6-dEB to a variety of useful

compounds. The compounds provided by the present invention can be provided to cultures of *Saccharopolyspora erythraea* and converted to the corresponding derivatives of erythromycins A, B, C, and D in accordance with the procedure provided in the Examples, below. To ensure that only the desired compound is produced, one can use an *S. erythraea eryA* mutant that is unable to produce 6-dEB but can still carry out the desired conversions (Weber *et al.*, 1985, *J. Bacteriol. 164*(1): 425-433). Also, one can employ other mutant strains, such as *eryB*, *eryC*, *eryG*, and/or *eryK* mutants, or mutant strains having mutations in multiple genes, to accumulate a preferred compound. The conversion can also be carried out in large fermentors for commercial production. Each of the erythromycins A, B, C, and D has antibiotic activity, although erythromycin A has the highest antibiotic activity. Moreover, each of these compounds can form, under treatment with mild acid, a C-6 to C-9 hemiketal with motilide activity. For formation of hemiketals with motilide activity, erythromycins B, C, and D, are preferred, as the presence of a C-12 hydroxyl allows the formation of an inactive compound that has a hemiketal formed between C-9 and C-12.

Thus, the present invention provides the compounds produced by hydroxylation and glycosylation of the compounds of the invention by action of the enzymes endogenous to *Saccharopolyspora erythraea* and mutant strains of *S. erythraea*. Such compounds are useful as antibiotics or as motilides directly or after chemical modification. For use as antibiotics, the compounds of the invention can be used directly without further chemical modification. Erythromycins A, B, C, and D all have antibiotic activity, and the corresponding compounds of the invention that result from the compounds being modified by *Saccharopolyspora erythraea* also have antibiotic activity. These compounds can be chemically modified, however, to provide other compounds of the invention with potent antibiotic activity. For example, alkylation of erythromycin at the C-6 hydroxyl can be used to produce potent antibiotics (clarithromycin is C-6-O-methyl), and other useful modifications are described in, for example, Griesgraber *et al.*, 1996, *J. Antibiot. 49*: 465-477, Agouridas *et al.*, 1998, *J. Med. Chem. 41*: 4080-4100, U.S. Patent Nos. 5,770,579; 5,760,233; 5,750,510; 5,747,467; 5,747,466; 5,656,607; 5,635,485; 5,614,614; 5,556,118; 5,543,400; 5,527,780;

5,444,051; 5,439,890; and 5,439,889; and PCT publication Nos. WO 98/09978 and 98/28316, each of which is incorporated herein by reference.

For use as motilides, the compounds of the invention can be used directly without further chemical modification. Erythromycin and certain erythromycin analogs are potent agonists of the motilin receptor that can be used clinically as prokinetic agents to induce phase III of migrating motor complexes, to increase esophageal peristalsis and LES pressure in patients with GERD, to accelerate gastric emptying in patients with gastric paresis, and to stimulate gall bladder contractions in patients after gallstone removal and in diabetics with autonomic neuropathy. See Peeters, 1999, Motilide Web Site, http://www.med.kuleuven.ac.be/med/gih/motilid.htm, and Omura *et al.*, 1987, Macrolides with gastrointestinal motor stimulating activity, *J. Med. Chem. 30*: 1941-3). The corresponding compounds of the invention that result from the compounds of the invention being modified by *Saccharopolyspora erythraea* also have motilide activity, particularly after conversion, which can also occur *in vivo*, to the C-6 to C-9 hemiketal by treatment with mild acid. Compounds lacking the C-12 hydroxyl are especially preferred for use as motilin agonists. These compounds can also be further chemically modified, however, to provide other compounds of the invention with potent motilide activity.

Moreover, and also as noted above, there are other useful organisms that can be employed to hydroxylate and/or glycosylate the compounds of the invention. As described above, the organisms can be mutants unable to produce the polyketide normally produced in that organism, the fermentation can be carried out on plates or in large fermentors, and the compounds produced can be chemically altered after fermentation. In addition to *Saccharopolyspora erythraea*, *Streptomyces venezuelae*, *S. narbonensis*, *S. antibioticus*, *Micromonospora megalomicea*, *S. fradiae*, and *S. thermotolerans* can also be used. In addition to antibiotic activity, compounds of the invention produced by treatment with *M. megalomicea* enzymes can have antiparasitic activity as well. Thus, the present invention provides the compounds produced by hydroxylation and glycosylation by action of the enzymes endogenous to *S. erythraea*, *S. venezuelae*, *S. narbonensis*, *S. antibioticus*, *M. megalomicea*, *S. fradiae*, and *S. thermotolerans*.

The present invention also provides methods and genetic constructs for producing the glycosylated and/or hydroxylated compounds of the invention directly in the host cell of interest. Thus, the recombinant genes of the invention, which include recombinant *megAI*, *megAII*, and *megAIII* genes with one or more deletions and/or insertions, including replacements of a *megA* gene fragment with a gene fragment from a heterologous PKS gene, can be included on expression vectors suitable for expression of the encoded gene products in *Saccharopolyspora erythraea*, *Micromonospora megalomicea*, *S. venezuelae*, *S. narbonensis*, *S. antibioticus*, *S. fradiae*, and *S. thermotolerans*.

The compounds of the invention can be produced by growing and fermenting the host cells of the invention under conditions known in the art for the production of other polyketides. The compounds of the invention can be isolated from the fermentation broths of these cultured cells and purified by standard procedures. The compounds can be readily formulated to provide the pharmaceutical compositions of the invention. The pharmaceutical compositions of the invention can be used in the form of a pharmaceutical preparation, for example, in solid, semisolid, or liquid form. This preparation will contain one or more of the compounds of the invention as an active ingredient in admixture with an organic or inorganic carrier or excipient suitable for external, enteral, or parenteral application. The active ingredient may be compounded, for example, with the usual non-toxic, pharmaceutically acceptable carriers for tablets, pellets, capsules, suppositories, solutions, emulsions, suspensions, and any other form suitable for use.

The carriers which can be used include water, glucose, lactose, gum acacia, gelatin, mannitol, starch paste, magnesium trisilicate, talc, corn starch, keratin, colloidal silica, potato starch, urea, and other carriers suitable for use in manufacturing preparations, in solid, semi-solid, or liquified form. In addition, auxiliary stabilizing, thickening, and coloring agents and perfumes may be used. For example, the compounds of the invention may be utilized with hydroxypropyl methylcellulose essentially as described in U.S. Patent No. 4,916,138, incorporated herein by reference, or with a surfactant essentially as described in EPO patent publication No. 428,169, incorporated herein by reference.

Oral dosage forms may be prepared essentially as described by Hondo *et al.*, 1987, *Transplantation Proceedings XIX*, Supp. 6: 17-22, incorporated herein by reference. Dosage forms for external application may be prepared essentially as described in EPO patent publication No. 423,714, incorporated herein by reference. The active compound is included in the pharmaceutical composition in an amount sufficient to produce the desired effect upon the disease process or condition.

For the treatment of conditions and diseases caused by infection, a compound of the invention may be administered orally, topically, parenterally, by inhalation spray, or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvant, and vehicles. The term parenteral, as used herein, includes subcutaneous injections, and intravenous, intramuscular, and intrasternal injection or infusion techniques.

Dosage levels of the compounds of the invention are of the order from about 0.01 mg to about 50 mg per kilogram of body weight per day, preferably from about 0.1 mg to about 10 mg per kilogram of body weight per day. The dosage levels are useful in the treatment of the above-indicated conditions (from about 0.7 mg to about 3.5 mg per patient per day, assuming a 70 kg patient). In addition, the compounds of the invention may be administered on an intermittent basis, i.e., at semi-weekly, weekly, semi-monthly, or monthly intervals.

The amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated and the particular mode of administration. For example, a formulation intended for oral administration to humans may contain from 0.5 mg to 5 gm of active agent compounded with an appropriate and convenient amount of carrier material, which may vary from about 5 percent to about 95 percent of the total composition. Dosage unit forms will generally contain from about 0.5 mg to about 500 mg of active ingredient. For external administration, the compounds of the invention may be formulated within the range of, for example, 0.00001% to 60% by weight, preferably from 0.001% to 10% by weight, and most preferably from about 0.005% to 0.8% by weight.

It will be understood, however, that the specific dose level for any particular patient will depend on a variety of factors. These factors include the

activity of the specific compound employed; the age, body weight, general health, sex, and diet of the subject; the time and route of administration and the rate of excretion of the drug; whether a drug combination is employed in the treatment; and the severity of the particular disease or condition for which therapy is sought.

A detailed description of the invention having been provided above, the following examples are given for the purpose of illustrating the invention and shall not be construed as being a limitation on the scope of the invention or claims.

Example 1

Cloning and Characterization of the Megalomicin Biosynthetic Gene Cluster from *Micromonospora meglomicea*

Experimental Procedures

Bacterial Strains, Media, and Growth Conditions

Routine DNA manipulations were performed in *Escherichia coli* XL1 Blue or *E. coli* XL1 Blue MR (Stratagene) using standard culture conditions (Sambrook *et al.*, 1989). *M. megalomicea* subs. *nigra* NRRL3275 was obtained from the ATCC collection and cultured according to recommended protocols. For isolation of genomic DNA, *M. megalomicea* was grown in TSB (Hopwood *et al.*, 1985) at 30 °C. *S. lividans* K4-114 (Ziermann and Betlach, 1999), which carries a deletion of the actinorhodin biosynthetic gene cluster, was used as the host for expression of the *megAI-AIII* genes. *S. lividans* strains were maintained on R5 agar at 30°C and grown in liquid YEME for preparation of protoplasts (Hopwood *et al.*, 1985). *S. erythraea* NRRL2338 was used for expression of the megosamine genes. *S. erythraea* strains were maintained on R5 agar at 34°C and grown in liquid TSB for preparation of protoplasts.

Manipulation of DNA and Organisms

Manipulation and transformation of DNA in *E. coli* was performed by standard procedures (Sambrook *et al.*, 1989) or by suppliers protocols. Protoplasts of *S. lividans* and *S. erythraea* were generated for transformation by plasmid DNA using the standard procedure. *S. lividans* transformants were selected on R5 using 2 ml of a 0.5 mg/ml thiostrepton overlay. *S. erythraea* transformants were selected on R5 using 1.5 ml of a 0.6 mg/ml apramycin overlay.

Isolation of the meg gene cluster

A cosmid library was prepared in SuperCos (Stratagene) from *M. megalomicea* total DNA partially digested with *Sau*3A I, and introduced into *E. coli* using a Gigapack III XL (Stratagene) *in-vitro* packaging kit. ^{32}P-labelled DNA probes encompassing the KS2 domain from *ery* DEBS, or a mixture of segments encompassing modules 1 and 2 from *ery* DEBS were used separately to screen the cosmid library by colony hybridization. Several colonies which hybridized with the probes were further analyzed by sequencing the ends of their cosmid inserts using T3 and T7 primers. BLAST (Altschul *et al.*, 1990) analysis of the sequences revealed several colonies with DNA sequences highly homologous to genes from the ery cluster. Together with restriction analysis, this led to the isolation of two overlapping cosmids, pKOS079-93A and pKOS079-93D which covered ~45 kb of the *meg* cluster. A 400 bp PCR fragment was generated from the left end of and pKOS079-93D and used to reprobe the cosmid library. Likewise, a 200 bp PCR fragment generated from the right end of pKOS079-93A was used to reprobe the cosmid library. Analysis of hybridizing colonies as described above resulted in identification of two additional cosmids, pKOS079-138B and pKOS79-124B which overlap the previous two cosmids. BLAST analysis of the far left and right end sequences of these cosmids indicated no homology to any known genes related to polyketide biosynthesis and therefore indicates that the set of four cosmids spans the entire megalomicin biosynthetic gene cluster.

DNA sequencing and analysis

PCR-based double stranded DNA sequencing was performed on a Beckman CEQ 2000 capillary sequencer using reagents and protocols provided by the manufacturer. A shotgun library of the entire cosmid pKOS079-93D insert was made as follows: DNA was first digested with *Dra* I to eliminate the vector fragment, then partially digested with *Sau*3A I. After agarose electrophoresis, bands between 1-3 kb were excised from the gel and ligated with *Bam*H I digested pUC19. Another shotgun library was generated from a 12 kb *Xho* I/*Eco*R I fragment subcloned from cosmid pKOS079-93A to extend the sequence to the *megF* gene. A 4 kb *Bgl* II/ *Xho* I fragment from cosmid pKOS079-138B was

sequenced by primer walking to extend the sequencing to the *megT* gene. Sequence was assembled using Sequencher (Gene Codes Corp.) software package and analyzed with MacVector (Oxford Molecular Group) and the NCBI BLAST server (www.ncbi.nlm.nih.gov/BLAST/).

Plasmids

Plasmid pKOS108-6 is a modified version of pKAO127'kan' (Ziermann and Betlach, 1999; Ziermann and Betlach, 2000) in which the *ery*AI-III genes between the *Pac* I and *Eco*R I sites have been replaced with the *meg*AI-III genes. This was done by first substituting a synthetic nucleotide DNA duplex (5'-TAAGAATTCGGAGATCTGGCCTCAGCTCTAGAC (SEQ ID NO: 21), complementary oligo 5'-AATTGTCTAGAGCTGAGGCCAGATCTCCGAATTCTTAAT (SEQ ID NO: 22)) between the *Pac* I and *Eco*R I sites of the pKAO127'kan' vector fragment. The 22 kb *Eco*R I/*Bgl* II fragment from cosmid pKOS079-93D containing the *megAI-II* genes was inserted into *Eco*R I and *Bgl* II sites of the resulting plasmid to generate pKOS024-84. A 12 kb *Bgl* II/*Bbv*C I fragment containing the *megAIII* and part of the *megCII* gene was subcloned from pKOS079-93A and excised as a *Bgl* II/*Xba* I fragment and ligated into the corresponding sites of pKOS024-84 to yield the final expression plasmid pKOS108-06.

The megosamine integrating vector, pKOS97-42, was constructed as follows: A subclone was generated containing the 4 kb *Xho* I/*Sca* I fragment from pKOS79-138B together with the 1.7 kb *Sca* I/*Pst* I fragment from pKOS79-93D in Litmus 28 (Stratagene). The entire 5.7 kb fragment was then excised as a *Spe* I/*Pst* I fragment and combined with the 6.3 kb *Pst* I/*Eco*R I fragment from KOS79-93D and *Eco*R I/*Xba* I digested pSET152 (Bierman *et al.*, 1992) to construct plasmid pKOS97-42.

Production and analysis of secondary metabolites

Fermentation for production of polyketide, LC/MS analysis, and quantification of 6-dEB for *S. lividans* K4-114/pKOS108-6 and *S. lividans* K4-114/pKAO127'kan' were essentially as previously described (Xue *et al.*, 1999). *S. erythraea* NRRL2338 and *S. erythraea*/pKOS97-42 were grown for 6 days in F1

media (Brünker *et al.*, 1998). Samples of broth were clarified in a microcentrifuge (5 min, 13,000 rpm). For LC/MS preparation, isopropanol was added to the supernatant (1:2 ratio) and centrifuged again. Erythromycins and megalomicins were detected by electrospray mass spectrometry and quantity was determined by evaporative light scattering detection (ELSD). The LC retention time and mass spectra of erythromycin and megalomicins were identical to known standards.

Nucleotide sequence of the meg *gene cluster*

A series of 4 overlapping inserts containing the *meg* cluster (Figure 9) were isolated from a cosmid library prepared from total genomic DNA of *M. megalomicea* and covers > 100 kb of the genome. A contiguous 48 kb segment which encodes the megalomicin PKS and several deoxysugar biosynthetic genes was sequenced and analyzed. The segment contains 17 complete ORFs as well as an incomplete ORF at each end, organized as shown in Figure 9.

PKS genes. The ORFs *megAI*, *megAII* and *megAIII* encode the polyketide synthase responsible for synthesis of 6-dEB. The enzyme complex, *meg* DEBS, is highly similar to *ery* DEBS, with each of the three predicted polypeptides sharing an average of 83% overall similarity with their *ery* PKS counterpart. Both PKSs are composed of 6 modules (2 modules per polypeptide) and each module is organized in the identical manner (Figure 9). A dendrogram analysis (Schwecke *et al.*, 1995) employing 70 acyltranferase (AT) domains revealed that the 6 *meg* extender AT domains cluster with AT domains that incorporate methylmalonyl CoA (not shown). The loading module of *meg* DEBS also lacks a KS^Q domain which is utilized by most macrolide PKSs for decarboxylation of the starter unit to initiate polyketide synthesis (Bisang *et al.*, 1999; Kuhstoss *et al.*, 1996; Kakavas *et al.*, 1997; Xue *et al.*, 1998), implying that priming begins with a propionate unit. In addition, a conserved Gly to Pro substitution in the NADPH-binding region of the ketoreductase (KR) domain of module 3 is observed in *meg* DEBS, which has been proposed to account for its inactivity in *ery* DEBS (Donadio *et al.*, 1991).

Deoxysugar genes. BLAST (Altschul *et al.*, 1990) analysis of the genes flanking the PKS indicated that 12 complete ORFs and 1 partial ORF appear to encode functions required for synthesis of one of the three megalomicin deoxysugars. Assignment of each ORF to a specific deoxysugar pathway was

made based on comparison to the *ery* genes and other related genes involved in deoxysugar biosynthesis (Table 2).

Table 2. Deduced functions of genes identified in the megalomicin gene cluster.

Gene	*Closest Match (polypeptide)*[a]	*% Sim*[a]	*Proposed Pathway*	*Proposed Function*	*Reference*
megT	EryBVI		Mycarose/ Megosamine	2,3-Dehydratase	(Summers *et al.*, 1997; Gaisser *et al.*, 1997)
megDVI	EryCII	63	Megosamine	3,4-Isomerase	(Summers *et al.*, 1997)
megDI	EryCIII	79	Megosamine	Glycosyltransferase	(Summers *et al.*, 1997)
megY	AcyA (*S. thermotolerans*)	52		Mycarose *O*-acyl-transferase	(Arisawa *et al.*, 1994)
megDII	EryCI	58	Megosamine	Aminotransferase	(Dhillon *et al.*, 1989; Summers *et al.*, 1997)
megDIII	DesVI (*S. venezuelae*)	61	Megosamine	Dimethyltransferase	(Xue *et al.*, 1998)
megDIV	DmnU (*S. peucetius*)	65	Megosamine	3,5-Epimerase	(Olano *et al.*, 1999)
megDV	Dehydrogenase (*A. orientalis*)	61	Megosamine	4-Ketoreductase	(Summers *et al.*, 1997; van Wageningen *et al.*, 1998)
megDVII	EryBII	73	Megosamine	2,3-Reductase	(Summers *et al.*, 1997)
megBV	EryBV	86	Mycarose	Glycosyltransferase	(Summers *et al.*, 1997; Gaisser *et al.*, 1997)
megBIV	EryBIV	80	Mycarose	4-Ketoreductase	(Summers *et al.*, 1997; Gaisser *et al.*, 1997)
megAI	EryAI	81	6-dEB	Polyketide Synthase	(Donadio and Katz, 1992)
megAII	EryAII	85	6-dEB	Polyketide Synthase	(Donadio and Katz, 1992)
megAIII	EryAIII	83	6-dEB	Polyketide Synthase	(Donadio and Katz, 1992)
megCII	EryCII	82	Desosamine	3,4-Isomerase	(Summers *et al.*, 1997)
meg CIII	EryCIII	89	Desosamine	Glycosylyltransferase	(Summers *et al.*, 1997)
megBII	EryBII	87	Mycarose	2,3-Reductase	(Summers *et al.*, 1997)
megH	EryH	84		Thioesterase	(Haydock *et al.*, 1991)
megF	EryF			C-6 Hydroxylase	(Weber *et al.*, 1991)

a. Determined by BLASTX analysis using default parameters.

Three ORFs, *megBV*, *megCIII* and *megDI*, encode glycosyltransferases, apparently one for attachment of each deoxysugar to the macrolide. MegBV was most similar to EryBV, the erythromycin mycarosyltransferase, and hence was assigned to the mycarose pathway in the *meg* cluster. The closest match for both of the remaining glycosyltransferases was EryCIII, the desosaminyltransferase in erythromycin biosynthesis. Given the higher degree of similarity between EryCIII and MegCIII (Table 2), MegCIII was designated the desosaminyltransferase, leaving MegDI as the proposed megosaminyltransferase. In similar fashion, assignments were made accordingly for; MegCII and MegDVI, two putative 3,4-isomerases similar to EryCII; MegBII and MegDVII, 2,3-reductases homologous to EryBII; MegBIV and MegDV, putative 4-ketoreductases similar to EryBIV (Table 2). The remaining ORFs involved in deoxysugar biosynthesis, *megT*, *megDII*, *megDIII* and *megDIV*, each encode a putative 2,3-dehydratase, aminotransferase, dimethyltransferase and 3,5-epimerase, respectively (Table 2). Since both the megosamine and desosamine pathways require an aminotransferase and a dimethyltransferase, and since mycarose and megosamine each require a 2,3-dehydratase and a 3,5-epimerase, assignments of these four genes to a specific pathway could not be made on the basis of sequence comparison alone. However, the latter three are implicated in megosamine biosynthesis by experiments described below.

Other genes. Two additional complete ORFs, designated *megY* and *megH* and an incomplete ORF, designated *megF*, were also identified in the cluster. MegH and MegF share high degrees of similarity with EryH and EryF. EryH and homologs in other macrolide gene clusters are thioesterase-like proteins with unknown function in polyketide gene clusters (Haydock *et al.*, 1991; Xue *et al.*, 1998; Butler *et al.*, 1999; Tang *et al.*, 1999). EryF encodes the erythronolide B C-6 hydroxylase (Figure 8) (Weber *et al.*, 1991; Andersen and Hutchinson, 1992). MegY does not have an *ery* counterpart but appears to belong to a (small) family of *O*-acyltransferases that transfer short acyl chains to macrolides. Two classes exist: AcyA and MdmB transfer acetyl or propionyl groups to the C-3 hydroxyls on 16-membered macrolide rings (Arisawa *et al.*, 1994; Hara and Hutchinson, 1992); CarE and Mpt transfer isovalerate or propionate to the mycarosyl moiety of carbomycin and midecamycin, respectively (Epp *et al.*, 1989; Arisawa *et al.*, 1993;

Gu *et al.*, 1996). The structures of various megalomicins suggest that MegY belongs to the latter class and is the acyltransferase which converts megalomicin A to megalomicins B, C1, or C2 (verified experimentally below).

Heterologous expression of the meg *PKS genes.*

The wild type and genetically modified versions of the *ery* DEBS have been used extensively in heterologous *Streptomyces* hosts for enzyme studies and the production of novel polyketide compounds. Given the similarities between the *ery* and *meg* DEBSs, production characteristics were compared in a commonly used *Streptomyces* host strain. The three *meg*A ORFs were cloned into the expression plasmid pKAO127'kan' (Ziermann and Betlach, 1999) in place of the *ery*A ORFs. Both plasmids, pKAO127'kan' encoding *ery* DEBS and pKOS108-06 encoding *meg* DEBS, were introduced in *Streptomyces lividans* K4-114 and the production of 6-dEB was determined in shake-flask fermentations. The production profiles were similar in both cases and the maximum titer of 6-dEB was between 30-40 mg/L. In addition, both PKSs produced small amounts (~5%) of 8,8a-deoxyoleandolide, which results from the priming of the PKS with acetate instead of propionate (Kao *et al.*, 1994b). This observation indicates that the loading AT domains of the PKSs display similar relaxed specificities towards starter units.

Conversion of erythromycin to megalomicin in S. erythraea.

An examination of the *meg* cluster revealed that the putative megosamine biosynthetic genes are clustered directly upstream of the PKS genes. If the hypothesis that these genes are sufficient for biosynthesis and attachment of megosamine to an erythromycin intermediate is correct, then functional expression of these genes in a strain which produces erythromycin, such as *S. erythraea*, should result in production of megalomicin. A 12 kb DNA fragment carrying all the genes between the leftmost *Xho*I site and the *Eco*RI site (Figure 9) was integrated in the chromosome of *S. erythraea* using the site-specific integrating vector pSET152 (Bierman *et al.*, 1992). It was surmised that the left and right ends of this fragment would contain necessary promoter regions for transcription of the convergent set of genes in *M. megalomicea* and that they would likely operate in *S. erythraea*.

Fermentation broth from *S. erythraea*/KOS97-42, which contains the integrated *meg* genes, was analyzed by LC/MS and compared to LC/MS profiles of the parent *S. erythraea* strain without the *meg* genes, as well as to megalomicin standards purified from *M. megalomicea*. The new strain was found to produce a mixture of erythromycin A and various megalomicins (~4:1 ratio), thereby showing that the predicted megosamine biosynthetic and glycosyltransferase genes are contained within the cloned *meg* fragment. The two most abundant congeners identified were megalomicins B and C1. Megalomicin A and C2 were also detected in smaller amounts. The presence of the megalomicins B, C1 and C2 also provides direct evidence for the function of the *O*-acyl transferase, MegY, which is present in the integrated *meg* fragment.

Discussion

The homologies observed among modular PKSs enabled the use of *ery* PKS genes to clone the *meg* biosynthetic gene cluster from *M. megalomicea*. The close similarities between the megalomicin and erythromycin biosynthetic pathways is also reflected in the overall organization of their genes and in the high degree of homology of the corresponding individual gene-encoded polypeptides. Production of 6-dEB from *meg* DEBS in *S. lividans* and conversion of erythromycin to megalomicin using the *megD* genes in *S. erythraea* provides direct evidence that the identified gene cluster is responsible for synthesis of megalomicin.

As seen in Figure 9, the ~ 40 kb segments of the two clusters beginning with *ery/megBV* on the left through the *ery/megF* genes retain a nearly identical organizational arrangement. The notable differences in this region are *eryG* and IS*1136* which are absent from the segment of the *meg* cluster analyzed. The *eryG* gene encodes an S-adenosylmethionine (SAM)-dependent mycarosyl methyltransferase that converts erythromycin C to erythromycin A (Figure 8) (Weber *et al.*, 1990; Haydock *et al.*, 1991). The mycarose moiety is modified by esterification (MegY) in megalomicin biosynthesis (Figure 8) and, therefore, the absence of an *eryG* homolog would be expected in the *meg* cluster. The IS*1136* element located between *eryAI* and *eryAII* (Donadio and Staver, 1993) is not

known to play a role in erythromycin biosynthesis and its origin in the *ery* cluster has not been determined.

Upstream of the common *meg/eryBIV* and *BV* genes, the gene clusters diverge. The ~ 6 kb segment between *eryBV* and *eryK*, the left border of the *ery* gene cluster (Pereda *et al.*, 1997), contains the remaining genes required for mycarose (*eryBVI* and *BVII*) and desosamine biosynthesis (*eryCIV, CV*, and *CVI*) and the C-12 hydroxylase (*eryK*) (Stassi *et al.*, 1993). In contrast, the region upstream of *megBV* encodes a set of genes (*megDI-DVII* and *megY*) which can account for all the activities unique to megalomicin biosynthesis (Figure 9). Since introduction of this *meg* DNA segment into *S. erythraea* results in production of megalomicins, it is clear that these genes encode the functions for TDP-megosamine biosynthesis and transfer to its putative substrate erythromycin C, and to acylate megalomicin A (Figure 8). The remaining region upstream of *megDVI* should therefore encode genes only for mycarose and desosamine biosynthesis.

Olano *et al.* (Olano *et al.*, 1999) have recently described a pathway for biosynthesis of TDP-L-daunosamine, a deoxysugar component of the antitumor compounds daunorubicin and doxorubicin produced by *Streptomyces peucetius*. Their pathway proposes four steps from the intermediate TDP-4-keto-6-deoxyglucose controlled by the gene cluster *dnmJQTUVZ*, although the functions for *dnmQ* and *dnmZ* could not be identified and the precise order of reactions in the pathway could not be determined. The genes *dnmT*, *dnmU*, *dnmJ* and *dnmV* each have proposed counterparts in the *meg* cluster, *megT*, *megDIV*, *megDII*, and *megDV*, respectively (see Figure 10)

It is possible to describe a pathway to convert TDP-2,6-dideoxy-3,4-diketo-D-hexose (or its enol tautomer), the last intermediate common to the mycarose and megosamine pathways, to TDP-megosamine through the sequence of 5-epimerization, 4-ketoreduction, 3-amination, and 3-*N*-dimethylation employing the genes *megDIV*, *megDV, megDII*, and *megDIII*. This employs the same functions proposed for biosynthesis of TDP-daunosamine by Olano *et al.*, but in a different sequential order. However, it does not account for the *megDVI* and *megDVII* genes since their activities are not required for this route. A parallel pathway which employs these genes is also shown in Figure 10. In this alternate route, 2,3-reduction and 3,4-tautomerization are performed by the *megDVII* and

megDVI gene products, respectively. A unified single pathway that employs both 4-ketoreduction (*megDV*) and 2,3-reduction (*megDVII*) could not be determined. Because the entire gene set from *megDVI* through *megDVII* was introduced in *S. erythraea* to produce TDP-megosamine, it is not possible to determine which, if either, of the two alternative pathways is operative, but this can be addressed through systematic gene disruption and complementation.

The 48 kb segment sequenced also contains genes required for synthesis of TDP-L-mycarose and TDP-D-desosamine (Fig 10). For the latter, *megCII*, which encodes a putative 3,4-isomerase, the first step in the committed TDP-desosamine pathway, appears to be translationally coupled to *megAIII*, almost exactly as its erythromycin counterpart, *eryCII*, was found translationally coupled to *eryAIII* (Summers *et al.*, 1997). The high degree of similarity between MegCII and EryCII suggests that the pathway to desosamine in the megalomicin- and erythromycin-producing organisms are most likely the same. Similarly, the finding that *megBII* and *megBIV*, encoding a 2,3-reductase and 4-ketoreductase, contain close homologs in the mycarose pathway for erythromycin also suggests that TDP-L-mycarose synthesis in the two host organisms is the same.

Of interest are the two genes that encode putative 2,3-reductases, *megBII* and *megDVII*. Because MegBII most closely resembles EryBII, a known mycarose biosynthetic enzyme (Weber *et al.*, 1990), and because *megBII* resides in the same location of the *meg* cluster as its counterpart in the *ery* cluster, *megBII* is assigned to the mycarose pathway and *megDVII* to the megosamine pathway. Furthermore, the lower degree of similarity between MegDVII and either EryBII or MegBII (Table 2) provides a basis for assigning the opposite L and D isomeric substrates to each of the enzymes (Figure 10). Finally, *megT*, which encodes a putative 2,3-dehydratase, is also related to a gene in the *ery* mycarose pathway, *eryBVI*. In *S. erythraea*, the proposed intermediate generated by EryBVI represents the first committed step in the biosynthesis of mycarose (Figure 10). However, the proposed pathways in Figure 10 suggest this may be an intermediate common to both mycarose and megosamine biosynthesis in *M. megalomicea*. Therefore, *megT* is named following the designation of the equivalent gene in the daunosamine pathway, *dnmT* (Olano *et al.*, 1999)

The preferred host-vector system for expression of *meg* DEBS described here has been used previously for the heterologous expression of modular PKS genes from the erythromycin (Kao *et al.*, 1994a; Ziermann and Betlach, 1999), picromycin (Tang *et al.*, 1999) and oleandomycin pathways, as well as for the generation of novel polyketide backbones where domains have been removed, added or exchanged in various combinations (McDaniel *et al.*, 1999). Recently, hybrid polyketides have been generated through the co-expression of subunits from different PKS systems (Tang *et al.*, 2000).

Expression of the *megDVI-megDVII* segment in *S. erythraea* and the corresponding production of megalomicins in this host establishes the likely order of sugar attachment in megalomicin synthesis. Furthermore, it provides a means to produce megalomicin in a more genetically friendly host organism, leading to the creation of megalomicin analogs by manipulating the PKS. Over 60 6-dEB analogs have been produced by combinatorial biosynthesis using the *ery* PKS (McDaniel *et al.*, 1999; Xue *et al.*, 1999). The titers of megalomicin could also be significantly increased above the 5 mg/L obtained from *M. megalomiciea* by introducing the genes into an industrially optimized strain of *S. erythraea*, many of which can produce as much as 10 g/L of erythromycin.

References

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Example 2

Stabilizing meg PKS Expression Plasmid by Codon Engineering

Materials and methods

All bacterial strains were cultured and transformed as described in Example 1.

Fermentation of Streptomyces and diketide feeding

Primary *Streptomyces* transformants were picked and placed in 6 mL of TSB liquid medium with 50 μg/L of thiostrepton and grown at 30°C. When the culture showed some growth (3-4days), it was transferred into a 250 mL flask containing 50 mL of R6 medium (pH 7.0) with 25 ug/L of thiostrepton and 1g/L of diketide ((2s,3R)2-methyl-3-hydroxyhexanoate N-propionyl cysteamine thioester) and placed in a 30°C incubator for 7 days.

Changing codons and making plasmids

There are several identical sequences in the coding seqüences for module 2 and module 6 of the megalomicin PKS gene cluster. Expression plasmids containing the full length megalomicin PKS appeared to be somewhat unstable and subject to deletion in $recA^+$ strains like ET124567 and *Streptomyces* by intra-plasmid homologous recombination. To prevent significant homologous recombination and so stabilize expression plasmids, the codons of two regions of the module 6 coding sequence that are identical to regions in the module 2 coding sequence were changed without changing the sequence of protein encoded. The two regions changed in module 6 were from the 26739th base to 27,267th base and from position 27,697th base to 27,987th base, which were identical to the region from position 6810th base to 7338thbase and regions from position 7778thbase to 8068th base, respectively. The start codon of the loading domain of the meg PKS was set to be the 1st base. These sequences are shown below

```
>  6810-7338 Sequence in Module 2
TTGCAGCGGTTGTCGGTGGCGGTGCGGAGGGGCGTCGGGTGTTGGGTGTGGTGGTGGGT
TCGGCGGTGAATCAGGATGGGGCGAGTAATGGGTTGGCGGCGCCGTCGGGGGTGGCGCAG
CAGCGGGTGATTCGGCGGGCGTGGGGTCGTGCGGGTGTGTCGGGTGGGGATGTGGGTGTG
GTGGAGGCGCATGGGACGGGGACGCGGTTGGGGGATCCGGTGGAGTTGGGGGCGTTGTTG
GGGACGTATGGGGTGGGTCGGGGTGGGGTGGGTCCGGTGGTGGTGGGTTCGGTGAAGGCG
AATGTGGGTCATGTGCAGGCGGCGGCGGGTGTGGTGGGTGTGATCAAGGTGGTGTTGGGG
TTGGGTCGGGGGTTGGTGGGTCCGATGGTGTGTCGGGGTGGGTTGTCGGGGTTGGTGGAT
TGGTCGTCGGGTGGGTTGGTGGTGGCGGATGGGGTGCGGGGGTGGCCGGTGGGTGTGGAT
GGGGTGCGTCGGGGTGGGGTGTCGGCGTTTGGGGTGTCGGGGACGAAT (SEQ ID NO: 23)
>  26736-27267 Sequence in Module 6
CTGCAGCGGTTGTCGGTGGCGGTGCGGAGGGGCGTCGGGTGTTGGGTGTGGTGGTGGGT
TCGGCGGTGAATCAGGATGGGGCGAGTAATGGGTTGGCGGCGCCGTCGGGGGTGGCGCAG
CAGCGGGTGATTCGGCGGGCGTGGGGTCGTGCGGGTGTGTCGGGTGGGGATGTGGGTGTG
GTGGAGGCGCATGGGACGGGGACGCGGTTGGGGGATCCGGTGGAGTTGGGGGCGTTGTTG
GGGACGTATGGGGTGGGTCGGGGTGGGGTGGGTCCGGTGGTGGTGGGTTCGGTGAAGGCG
AATGTGGGTCATGTGCAGGCGGCGGCGGGTGTGGTGGGTGTGATCAAGGTGGTGTTGGGG
```

TTGGGTCGGGGGTTGGTGGGTCCGATGGTGTGTCGGGGTGGGTTGTCGGGGTTGGTGGAT
TGGTCGTCGGGTGGGTTGGTGGTGGCGGATGGGGTGCGGGGGTGGCCGGTGGGTGTGGAT
GGGGTGCGTCGGGGTGGGGTGTCGGCGTTTGGGGTGTCGGGGACGAAT (SEQ ID NO: 24)
> 26736-27267 Sequence with Codon Changes
CTGCAGCGCCTCTCCGTCGCCGTCCGCGAGGGCCGCCGAGTCCTCGGCGTCGTCGTCGGC
TCGGCCGTCAACCAAGACGGCGCGTCAAACGGCCTCGCCGCGCCCTCCGGCGTCGCCCAG
CAGCGCGTCATACGCCGCGCGTGGGACGCGCCGGAGTATCGGGCGGCGACGTCGGAGTC
GTCGAGGCCCACGGCACCGGCACCCGCCTCGGGGATCCCGTCGAGCTGGGCGCCCTCCTG
GGCACGTACGGCGTCGGCCGCGGCGGCGTCGGCCCGGTCGTCGTCGGCAGCGTCAAGGCC
AACGTCGGCCACGTCCAGGCCGCGGCCGGCGTCGTCGGGGTCATCAAGGTCGTCCTCGGC
CTCGGCCGCGGGCTGGTCGGCCCGATGGTCTGCCGCGGCGGCCTCAGCGGCCTCGTCGAC
TGGTCGTCCGGCGGCCTGGTCGTCGCGGACGGGGTCCGCGGCTGGCCGGTCGGCGTCGAC
GGCGTCCGCCGGGGCGGCGTCTCGGCGTTCGGCGTCAGCGGGACGAAT (SEQ ID NO: 25)

> 6978-7337 Sequence in Module 2
GGTGGAGTGTGATGCGGTGGTGTCGTCGGTGGTGGGGTTTTCGGTGTTGGGGGTGTTGGA
GGGTCGGTCGGGTGCGCCGTCGTTGGATCGGGTGGATGTGGTGCAGCCGGTGTTGTTCGT
GGTGATGGTGTCGTTGGCGCGGTTGTGGCGGTGGTGTGGGGTTGTGCCTGCGGCGGTGGT
GGGTCATTCGCAGGGGGAGATCGCGGCGGCGGTGGTGGCGGGGGTGTTGTCGGTGGGTGA
TGGTGCGCGGGTGGTGGCGTTGCGGGCGCGGGCGTTGCGGGCGTTGGCCGG (SEQ ID NO: 26)
> 27697-27987 Sequence in Module 6
GGTGGAGTGTGATGCGGTGGTGTCGTCGGTGGTGGGGTTTTCGGTGTTGGGGGTGTTGGA
GGGTCGGTCGGGTGCGCCGTCGTTGGATCGGGTGGATGTGGTGCAGCCGGTGTTGTTCGT
GGTGATGGTGTCGTTGGCGCGGTTGTGGCGGTGGTGTGGGGTTGTGCCTGCGGCGGTGGT
GGGTCATTCGCAGGGGGAGATCGCGGCGGCGGTGGTGGCGGGGGTGTTGTCGGTGGGTGA
TGGTGCGCGGGTGGTGGCGTTGCGGGCGCGGGCGTTGCGGGCGTTGGCCGG (SEQ ID NO: 27)
> 27697-27987 Sequence with Codon Changes
CGTGGAGTGCGATGCGGTCGTGTCGAGCGTCGTCGGCTTCAGCGTGCTGGGCGTCCTGGA
GGGCCGCAGCGGCGCCCCGAGCCTGGACCGCGTCGACGTGGTCCAGCCGGTCCTGTTCGT
GGTCATGGTCAGCCTGGCCCGCCTGTGGCGCTGGTGCGGCGTGGTCCCGGCCGCCGTGGT
CGGCCACAGCCAGGGCGAGATCGCCGCCGCGGTCGTGGCCGGCGTCCTGAGCGTCGGCGA
CGGCGCCCGCGTCGTGGCCCTGCGCGCCCGCGCCCTGCGCGCCCTGGCCGG (SEQ ID NO: 28)

Three pieces of DNA from the two regions above were synthesized and verified by Retrogen, and the synthesized DNAs were cloned into pCR-Blunt II –TOPO, as shown in the Table 3 below.

Table 3. Plasmids containing synthesized DNA

Plasmids	Cloning sites and positions in meg PKS
pKOS97-1613	PstI-BamHI, 26,739th-26,947th base
PKOS97-1622	BamHI-BsmI, 26,947th –27,267th base
PKOS97-1628	SfaNI-FseI, 27,697th- 27,987th base

Assembly of the expression plasmid

First, ligation of the PstI-BamHI fragment of pKOS97-1613, the BamHI-BsmI fragment of pKOS97-1622 and BsmI-PstI linearized pKOS97-90 produced

pKOS97-151. Then, the insertion of the SfaNI-FseI fragment of pKOS97-1628 into pKOS97-151 gave rise to pKSO97-152. Then, the PstI-BlpI fragment of pKOS97-125 was used to replace the PstI-BlpI fragment of pKOS97-90a and produced pKOS97-160.

The final expression plasmid (in pRM5) pKOS97-162 was the result of BglII-NheI fragment of pKOS97-160 inserted into BglII-NheI sites of pKOS108-04.

Another expression plasmid pKOS97-152a was made by a four-fragment ligation. The four fragments were a BlpI-XbaI fragment (containing a cos site) of pKOS97-92a, a BglII-PstI fragment of pKOS97-81, a PstI-BlpI fragment of pKOS97-152, and a BglII-XbaI fragment of pKOS108-04 (as the vector).

Tests of the constructed plasmids showed that the plasmids containing the modified coding sequences were more stable than plasmids containing unmodified coding sequence.

Example 3

Construction of Ole-Meg Hybrid PKS

Construction of pRM1-based pKOS098-48 for the expression of OlePKS modules 1-4.

The 240-bp fragment containing the 3'-end portion of *oleAII* gene (at nt 11210-11452; the first base of the start codon of *oleAII* is nt 1) was PCR amplified with primers N98-38-1 (5'GAACAACTCCTGTCT<u>GCGGCCGC</u>G-3') (SEQ ID NO: 29) and N98-38-3 (5'-CG<u>GAATTC</u>**TCTAGA**GTCACGTCTCCAACCGCTTGTCGAGG-3') (SEQ ID NO: 30). The fragment contains a naturally occurring NotI site at its 5'-end and the engineered XbaI (bold) and EcoRI sites (underline) at its 3'-end following the *oleAII* stop codon. pKOS38-189 was digested with EcoRI and NotI to give five fragments of 8 kb, 5 kb, 4 kb, 2.5 kb and 2 kb. The 8-kb EcoRI-NotI fragment containing *oleAII* gene nt 2961 to nt 11210 and the 240-bp NotI, EcoRI treated PCR fragment were ligated into litmus 28 at the EcoRI site via a three-fragment ligation to give pKOS98-46. The 8.2-kb EoRI fragment from pKOS98-46 was cloned into pKOS38-174, a pRM1 derived plasmid containing *oleAI* and nt 1 to nt 2960 of *oleAII* to give pKOS98-48.

Construction of pSET152-based pKOS98-60 for the expression of megPKS modules 5-6.

The 360-bp fragment containing nt 1 to nt 366 of *megAIII* was PCR amplified with primers N98-40-3 (5'-TCTAGACTTAATTAAGGAGGACACATATGAGCGA-GAGCAGC-GGCATGACCG-3') (SEQ ID NO: 31) and N98-40-2 (5'- AACGCCTCCCAG-GAGATCTCCAGCA-3') (SEQ ID NO: 32). A PacI site and a NdeI site as well as the ribosome binding site were introduced at the 5'-end of the *megAI* start codon. The 360-bp PacI-BglII fragment was inserted into pKOS108-06 replacing the 22-kb PacI-BglII fragment to yield pKOS98-55. The 10-kb PacI-XbaI fragment containing megAIII gene and the annealed oligos N98-23-1 (5'-AATTCATAGCCTAGGT-3') (SEQ ID NO: 33) and N98-23-2 (5'-CTAGACCTAGGCTATG-3') (SEQ ID NO: 34) were ligated to PacI and EcoRI treated pSET152 derivative pKOS98-14 via a three-fragment ligation to give pKOS98-60.

Example 4

Conversion of Erythronolides to Erythromycins

A sample of a polyketide (~50 to 100 mg) is dissolved in 0.6 mL of ethanol and diluted to 3 mL with sterile water. This solution is used to overlay a three day old culture of *Saccharopolyspora erythraea* WHM34 (an *eryA* mutant) grown on a 100 mm R2YE agar plate at 30°C. After drying, the plate is incubated at 30°C for four days. The agar is chopped and then extracted three times with 100 mL portions of 1% triethylamine in ethyl acetate. The extracts are combined and evaporated. The crude product is purified by preparative HPLC (C-18 reversed phase, water-acetonitrile gradient containing 1% acetic acid). Fractions are analyzed by mass spectrometry, and those containing pure compound are pooled, neutralized with triethylamine, and evaporated to a syrup. The syrup is dissolved in water and extracted three times with equal volumes of ethyl acetate. The organic extracts are combined, washed once with saturated aqueous $NaHCO_3$, dried over Na_2SO_4, filtered, and evaporated to yield ~0.15 mg of product. The product is a glycosylated and hydroxylated compound corresponding to

erythromycin A, B, C, and D but differing therefrom as the compound provided differed from 6-dEB.

Example 5

Measurement of Antibacterial Activity

Antibacterial activity is determined using either disk diffusion assays with *Bacillus cereus* as the test organism or by measurement of minimum inhibitory concentrations (MIC) in liquid culture against sensitive and resistant strains of *Staphylococcus pneumoniae*.

Example 6

Evaluation of Antiparasitic Activity

Compounds can initially screened *in vitro* using cultures of *P. falciparum* FCR-3 and K1 strains, then *in vivo* using mice infected with *P. berghei*. Mammalian cell toxicity can be determined in FM3A or KB cells. Compounds can also be screened for activity against *P. berhei*. Compounds are also tested in animal studies and clinical trials to test the antiparasitic activity broadly (antimalarial, trypanosomiasis and Leishmaniasis).

The invention having now been described by way of written description and example, those of skill in the art will recognize that the invention can be practiced in a variety of embodiments and that the foregoing description and examples are for purposes of illustration and not limitation of the following claims.

Claims

1. An isolated nucleic acid comprising a nucleotide sequence encoding a domain of megalomicin polyketide synthase (PKS) or a megalomicin modification enzyme.

2. The isolated nucleic acid of claim 1, which encodes a PKS open reading frame (ORF) selected from the group consisting of megAI, megAII and megAIII.

3. The isolated nucleic acid of claim 1, wherein the PKS domain is selected from the group consisting of a TE domain, a KS domain, an AT domain, an ACP domain, a KR domain, a DH domain, and an ER domain.

4. The isolated nucleic acid of claim 1, wherein the nucleic acid comprises the coding sequence for a loading module, a thioesterase domain, and all six extender modules of megalomicin PKS.

5. The isolated nucleic acid of claim 1, which encodes a megalomicin modification enzyme that is involved in the conversion of 6-dEB into a megalomicin.

6. The isolated nucleic acid of claim 5, which encodes a megalomicin modification enzyme that is involved in the biosynthesis of mycarose, megosamine or desosamine.

7. The isolated nucleic acid of claim 1, wherein the nucleic acid codons of homologous regions within the PKS or the megalomicin modification enzyme coding sequence have been changed to reduce or abolish the homology without changing the amino acid sequences encoded by said changed nucleic acid codons.

8. The isolated nucleic acid of claim 1, which isolated nucleic acid fragment hybridizes to a nucleic acid having a nucleotide sequence set forth in the SEQ. ID NO:1.

9. A polypeptide, which is encoded by the isolated nucleic acid fragment of claim 1.

10. A recombinant DNA expression vector, comprising the isolated nucleic acid of claim 1 operably linked to a promoter.

11. A recombinant host cell, comprising the recombinant DNA expression vector of claim 10.

12. The recombinant host cell of claim 11, which is a *Streptomyces* or *Saccharopolyspora* host cell.

13. A recombinant host cell of claim 11, which comprises:

a) at least two separate autonomously replicating recombinant DNA expression vectors, each of said vectors comprises a recombinant DNA compound encoding a megalomicin PKS domain or a megalomicin modification enzyme operably linked to a promoter; or

b) at least one autonomously replicating recombinant DNA expression vector and at least one modified chromosome, each of said vector(s) and each of said modified chromosome comprises a recombinant DNA compound encoding a megalomicin PKS domain or a megalomicin modification enzyme operably linked to a promoter.

14. A hybrid PKS that comprises a polypeptide of claim 9 and is composed of at least a portion of a megalomicin PKS and at least a portion of a second PKS for a polyketide other than megalomicin.

15. The hybrid PKS of claim 14, wherein the second PKS is selected from the group consisting of a narbonolide PKS, an oleandolide PKS, and a DEBS PKS.

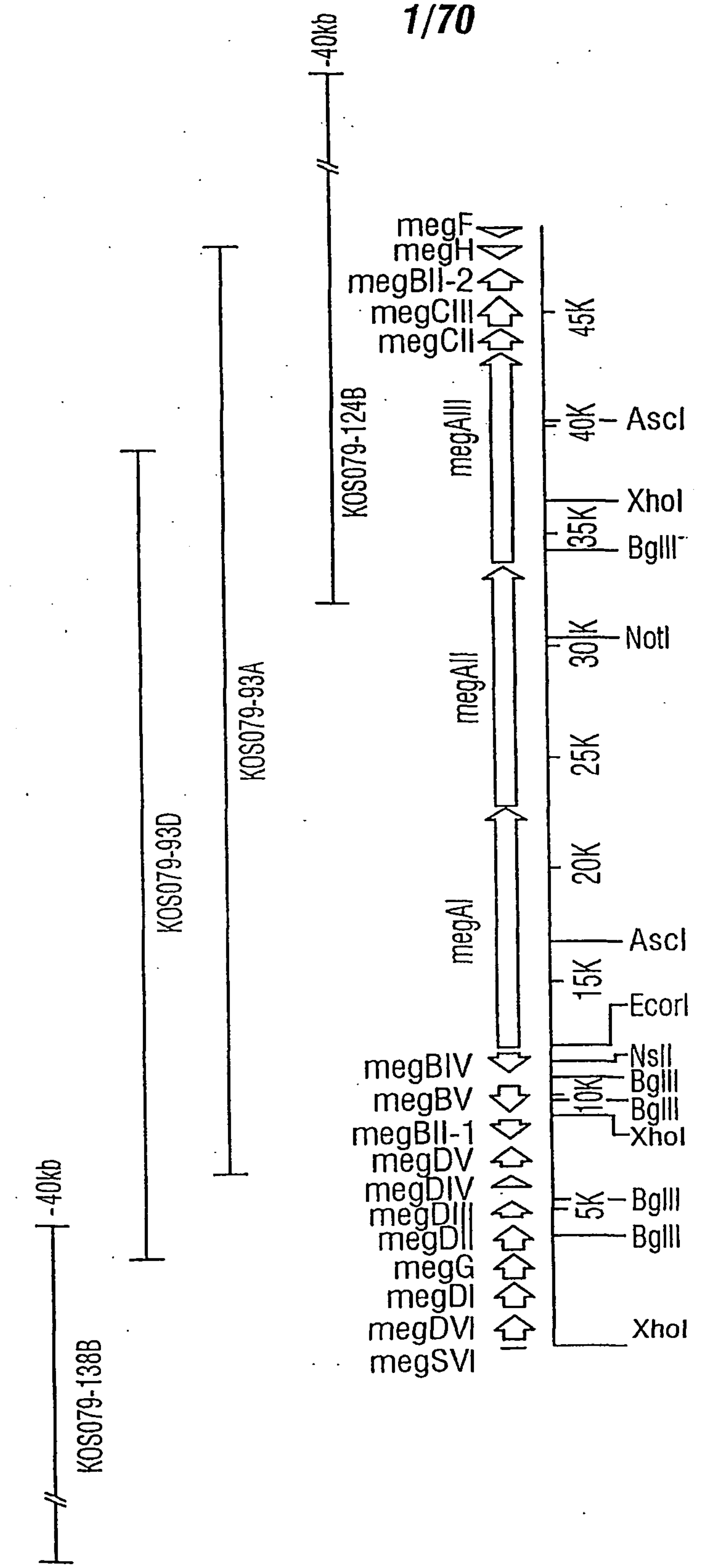
16. The hybrid PKS of claim 15 that is composed of the megAI and megAII gene products and the oleAIII gene product.

17. The hybrid PKS of claim 16, wherein the KS domain of module 1 of the megAI gene product has been inactivated by mutation.

18. A method of producing a polyketide, which method comprises growing the recombinant host cell of claim 11 under conditions whereby the megalomicin PKS domain encoded by the recombinant expression vector is produced and the polyketide is synthesized by the cell, and recovering the synthesized polyketide.

19. A recombinant host cell that comprises a recombinant expression vector that encodes a megalomicin modification enzyme.

20. The recombinant host cell of claim 19 that produces megosamine and can attach megosamine to a polyketide, wherein said host cell, in its naturally occurring non-recombinant state cannot produce megosamine.



megF
megH
megBII-2
megCIII
megCII
megAIII
megAII
megAI
megBIV
megBV
megBII-1
megDV
megDIV
megDIII
megDII
megG
megDI
megDVI
megSVI
45K
40K
35K
30K
25K
20K
15K
10K
5K
AscI
XhoI
BglII
NotI
AscI
EcorI
NsII
BglII
BglII
XhoI
BglII
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KOS079-124B
KOS079-93A
KOS079-93D
-40kb
KOS079-138B

FIG. 1

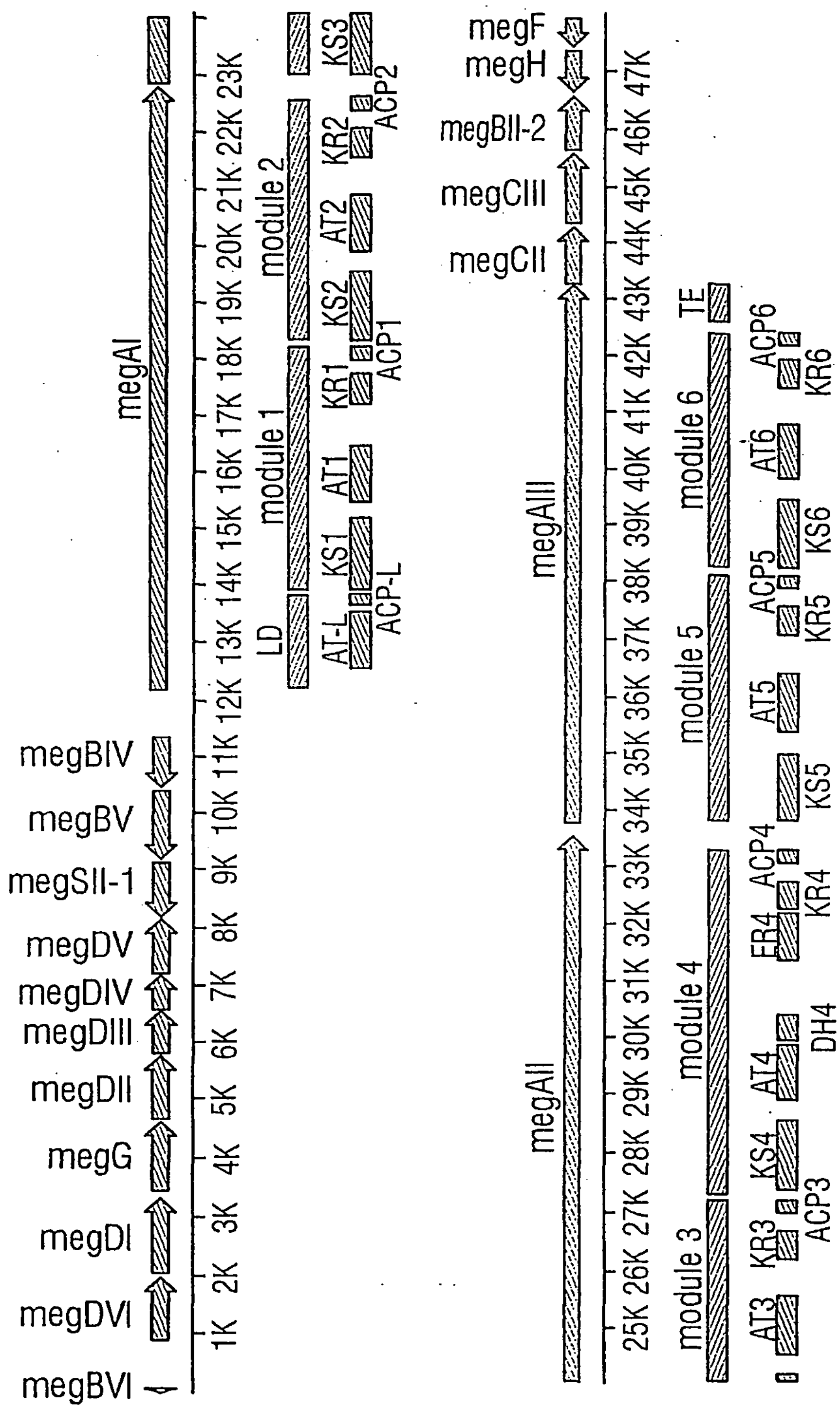
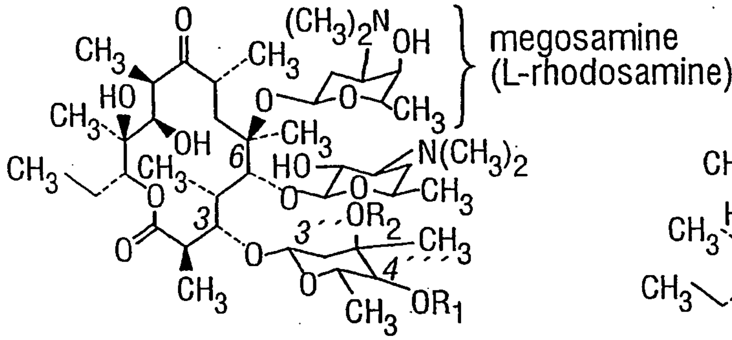
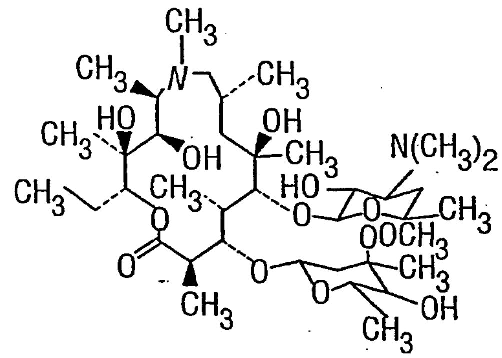

megAI
megBIV
megBV
megSII-1
megDV
megDIV
megDIII
megDII
megG
megDI
megDVI
megBVI
1K 2K 3K 4K 5K 6K 7K 8K 9K 10K 11K 12K 13K 14K 15K 16K 17K 18K 19K 20K 21K 22K 23K
LD
module 1
module 2
AT-L
ACP-L
KS1
AT1
KR1
ACP1
KS2
AT2
KR2
ACP2
KS3
megF
megH
megBII-2
megCIII
megCII
megAIII
megAII
25K 26K 27K 28K 29K 30K 31K 32K 33K 34K 35K 36K 37K 38K 39K 40K 41K 42K 43K 44K 45K 46K 47K
module 3
module 4
module 5
module 6
TE
AT3
KR3
ACP3
KS4
AT4
DH4
ER4
KR4
ACP4
KS5
AT5
KR5
ACP5
KS6
AT6
KR6
ACP6

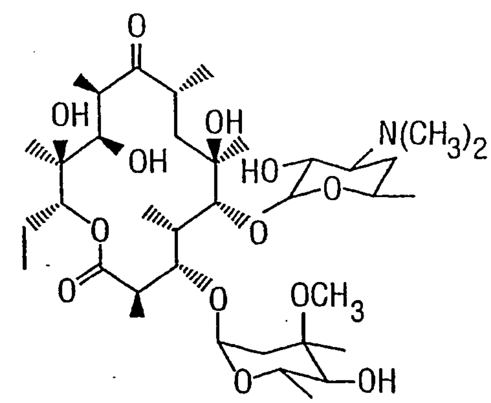
FIG. 2



		R_1	R_2
Megalomicin	A	H	H
	B	$COCH_3$	H
	C1	$COCH_3$	$COCH_3$
	C2	$COCH_2CH_3$	$COCH_3$



azithromycin
(Azithromax)



Erythromycin A

FIG. 3

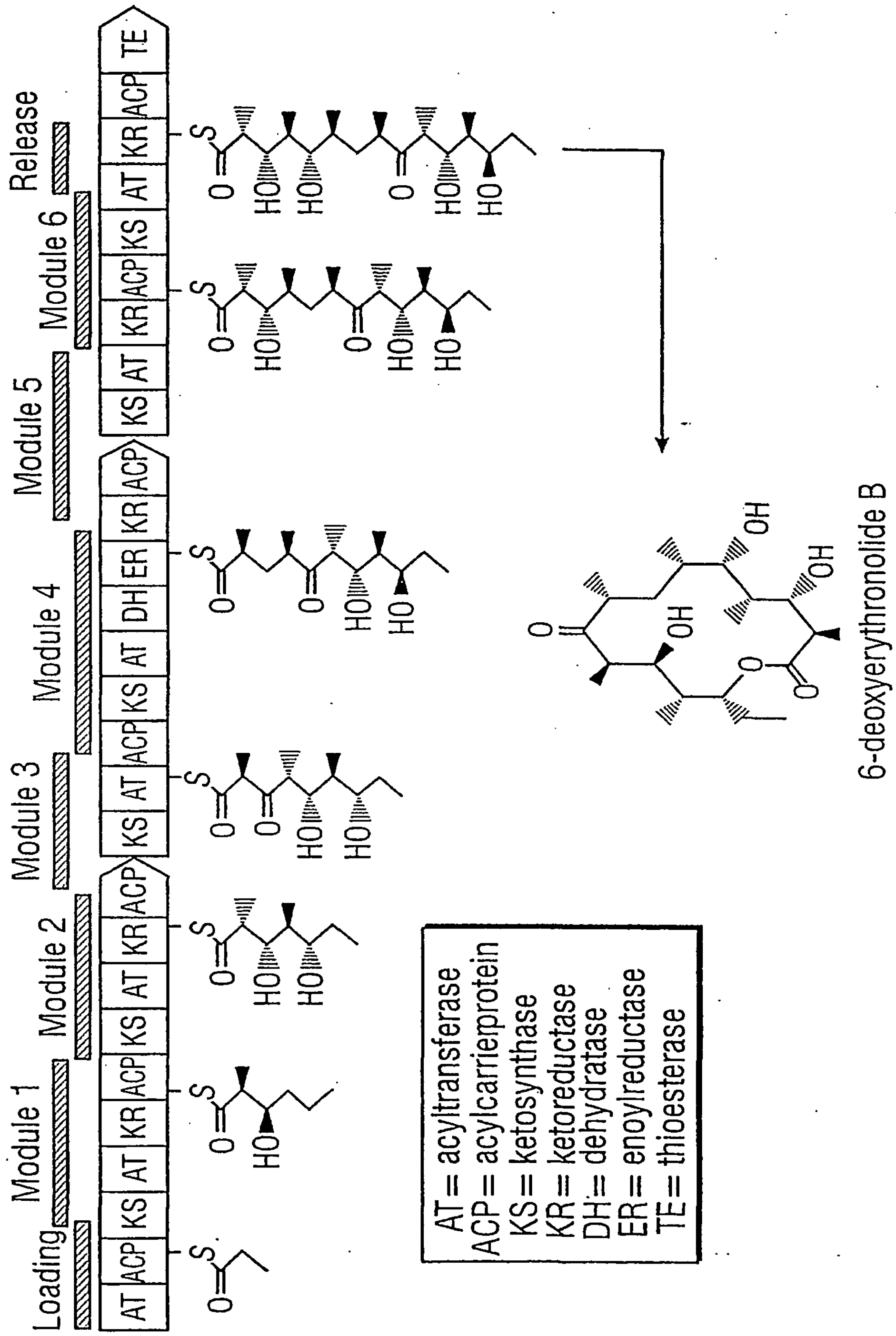

Loading
Module 1
Module 2
Module 3
Module 4
Module 5
Module 6
Release
AT ACP KS AT KR ACP KS AT KR ACP KS AT ACP KS AT DH ER KR ACP
KS AT KR ACP KS AT KR ACP TE
6-deoxyerythronolide B
AT = acyltransferase
ACP = acylcarrierprotein
KS = ketosynthase
KR = ketoreductase
DH = dehydratase
ER = enoylreductase
TE = thioesterase

FIG. 4

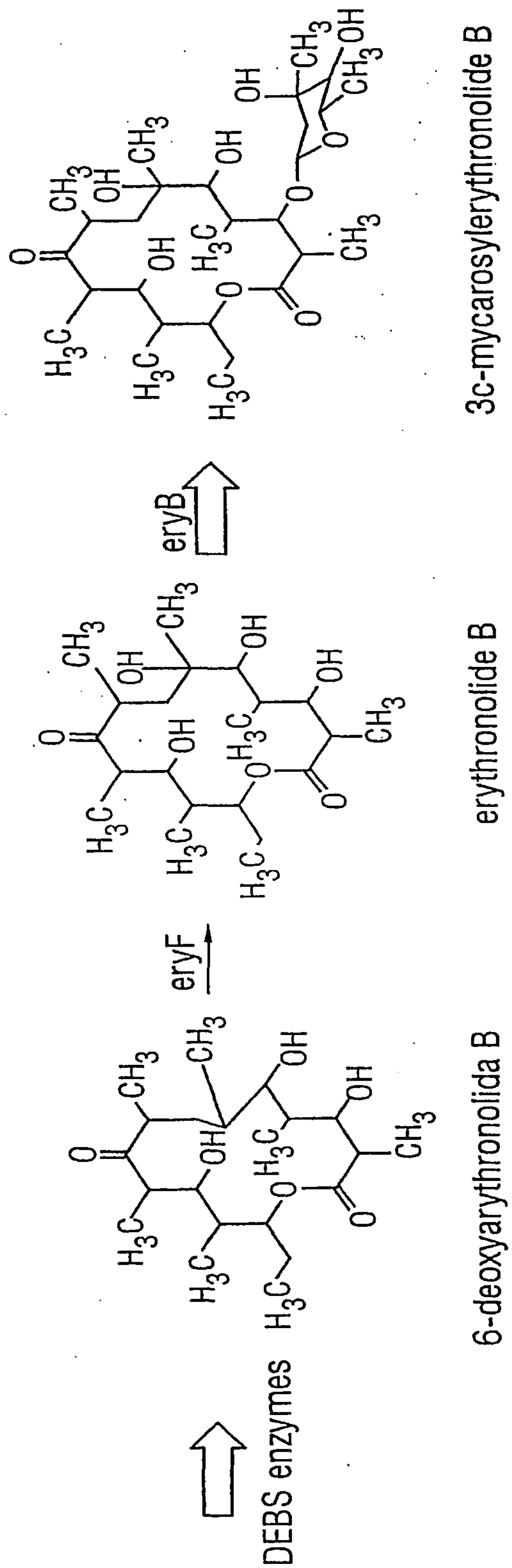
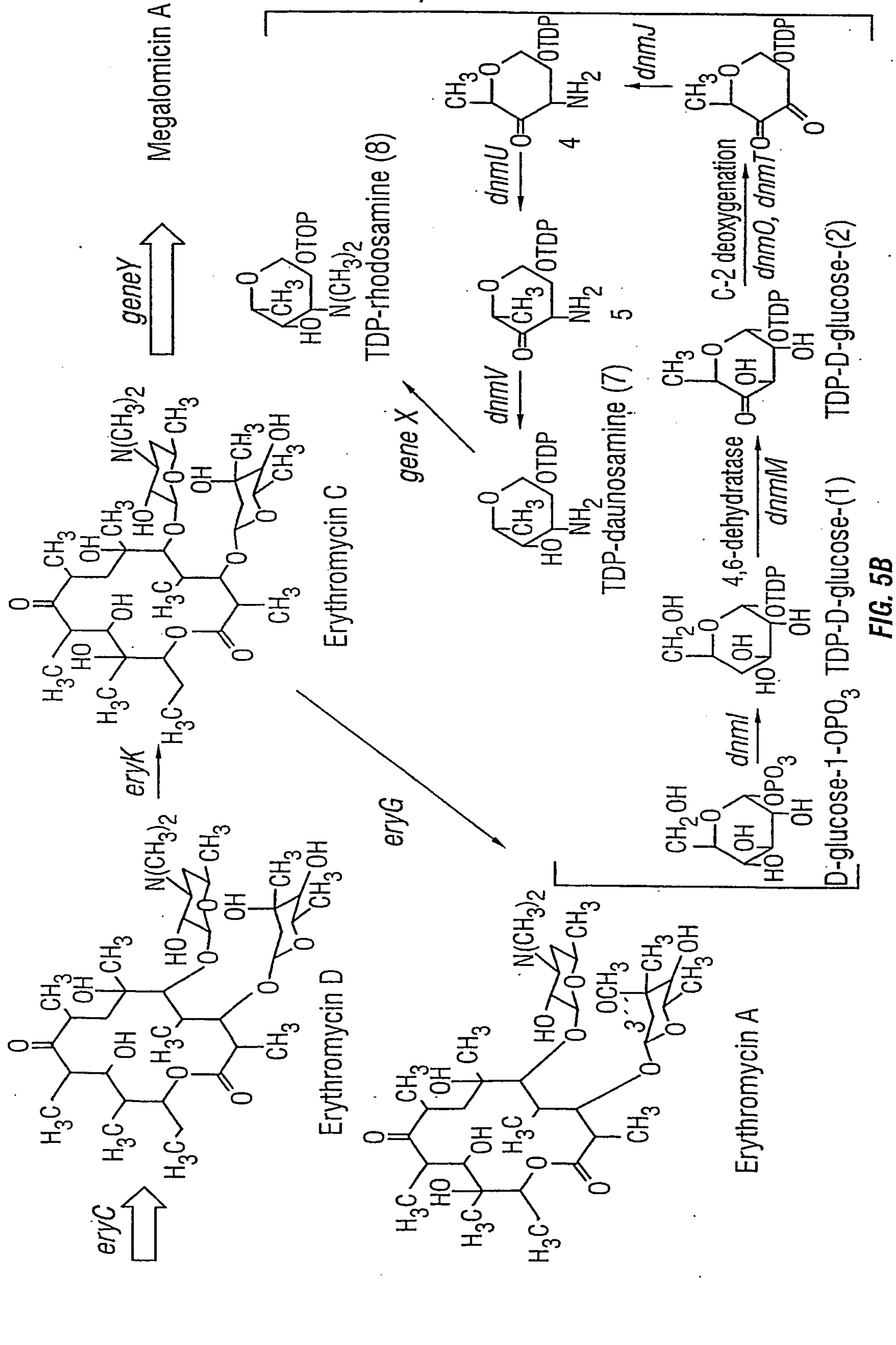

DEBS enzymes
6-deoxyarythronolida B
eryF
erythronolide B
eryB
3c-mycarosylerythronolide B

FIG. 5A



Megalomicin A
geneY
TDP-rhodosamine (8)
gene X
dnmU
dnmJ
dnmV
C-2 deoxygenation
dnmO, dnmT
TDP-daunosamine (7)
4,6-dehydratase
dnmM
TDP-D-glucose-(2)
TDP-D-glucose-(1)
dnmI
D-glucose-1-OPO$_3$
Erythromycin C
eryK
eryG
Erythromycin D
eryC
Erythromycin A

FIG. 5B

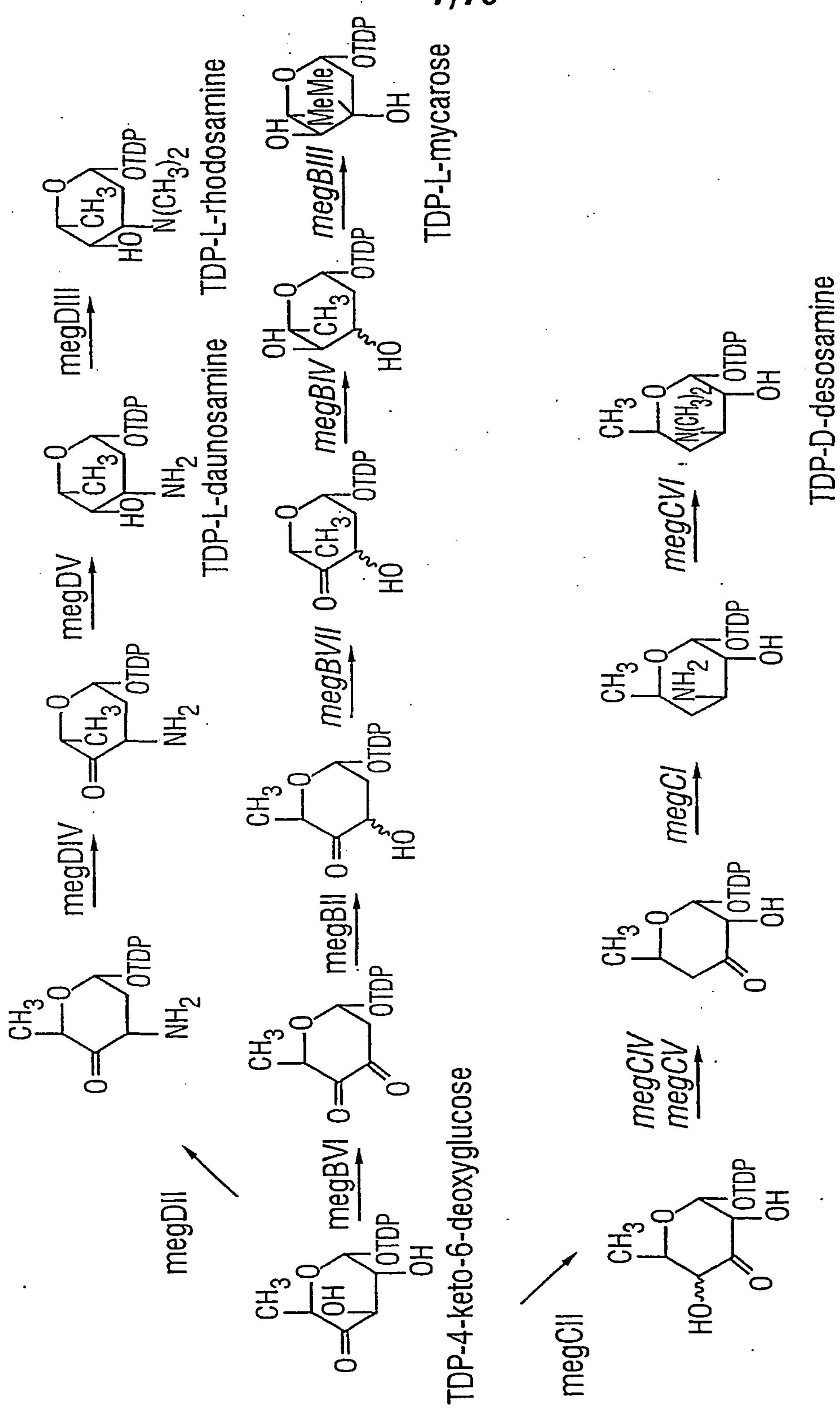

TDP-4-keto-6-deoxyglucose
megDII
megDIV
megDV
megDIII
TDP-L-daunosamine
TDP-L-rhodosamine
megBVI
megBII
megBVII
megBIV
megBIII
TDP-L-mycarose
megCII
megCIV
megCV
megCI
megCVI
TDP-D-desosamine

FIG. 6

```
LOCUS       1            47981 bp    DNA             01-MAY-2000
DEFINITION  Megalomicin biosynthetic gene cluster, polyketide synthase,
            desosamine, megosamine, and mycarose biosynthesis genes.
ACCESSION   1
VERSION
KEYWORDS
SOURCE      Micromonospora megalomicea.
  ORGANISM  Micromonospora megalomicea
            Unclassified.
REFERENCE   1  (bases 1 to 47981)
  AUTHORS   Volchegursky,Y., Hu,Z., Katz,L. and McDaniel,R.
  TITLE     Biosynthesis of the Anti-Parasitic Agent Megalomicin:
            Transformation of Erythromycin to Megalomicin in Sacharopolyspora
            erythraea
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 47981)
  AUTHORS   McDaniel,r. and Volchegursky,Y.
  TITLE     Direct Submission
  JOURNAL   Submitted (01-MAY-2000) Kosan Biosciences, Inc., 3828 Bay Center
            Place, Hayward, CA 94545, USA
FEATURES             Location/Qualifiers
     source          1..47981
                     /organism="Micromonospora megalomicea"
```

FIG. 7-1

```
                /strain="NRRL3275"
                /sub_species="nigra"
gene            complement (<1..144)
                /gene="megT"
CDS             complement (<1..144)
                /gene="megT"
                /codon_start=1
                /transl_table=11
                /product="TDP-4-keto-6-deoxyglucose-2,3-dehydratase"
                /translation="MGDRVNGHATPESTQSAIRFLTRHGGPPTATDDVHDWLAHRAAE
                HRLE" (SEQ ID NO: 2)
gene            928..2061
                /gene="megDVI"
CDS             928..2061
                /gene="megDVI"
                /codon_start=1
                /transl_table=11
                /product="TDP-4-keto-6-deoxyhexose 3,4-isomerase"
                /translation="MAVGDRRRLGRELQMARGLYWGFGANGDLYSMLLSGRDDDPWTW
                YERLRAAGRGPYASRAGTWVVGDHRTAAEVLADPGFTHGPPDAARWMQVAHCPAASWA
                GPFREFYARTEDAASVTVDADWLQQRCARLVTELGSRFDLVNDFAREVPVLALGTAPA
                LKGVDPDRLRSWTSATRVCLDAQVSPQQLAVTEQALTALDEIDAVTGGRDAAVLVGVV
                AELAANTVGNAVLAVTELPELAARLADDPETATRVVTEVSRTSPGVHLERRTAASDRR
```

FIG. 7-2

```
         VGGVDVPTGGEVTVVVAAANRDPEVFTDPDRFDVDRGGDAEILSSRPGSPRTDLDALV
         ATLATAALRAAAPVLPRLSRSGPVIRRRRSPVARGLSRCPVEL" (SEQ ID NO: 3)
gene     2072..3382
         /gene="megDI"
CDS      2072..3382
         /gene="megDI"
         /codon_start=1
         /transl_table=11
         /product="TDP-megosamine glycosyltransferase"
         /translation="MRVVFSSMAVNSHLFGLVPLASAFQAAGHEVRVVASPALTDDVT
         GAGLTAVPVGDDVELVEWHAHAGQDIVEYMRTLDWVDQSHTTMSWDDLLGMQTTFTPT
         FFALMSPDSLIDGMVEFCRSWRPDWIVWEPLTFAAPIAARVTGTPHARMLWGPDVATR
         ARQSFLRLLAHQEVEHREDPLAEWFDWTLRRFGDDPHLSFDEELVLGQWTVDPIPEPL
         RIDTGVRTVGMRYVPYNGPSVVPAWLLREPERRRVCLTLGGSSREHGIGQVSIGEMLD
         AIADIDAEFVATFDDQQLVGVGSVPANVRTAGFVPMNVLLPTCAATVHHGGTGSWLTA
         AIHGVPQIILSDADTEVHAKQLQDLGAGLSLPVAGMTAEHLRGAIERVLDEPAYRLGA
         ERMRDGMRTDPSPAQVVGICQDLAADRAARGRQPRRTAEPHLPR"(SEQ ID NO: 4)
gene     3462..4634
         /gene="megY"
CDS      3462..4634
         /gene="megY"
         /codon_start=1
         /transl_table=11
```

FIG. 7-3

```
                 /product="mycarose O-acyltransferase"
                 /translation="MVTSTNLDTTARPALNSLTGMRFVAAFLVFFTHVLSRLIPNSYV
                 YADGLDAFWQTTGRVGVSFFFILSGFVLTWSARASDSVWSFWRRRVCKLFPNHLVTAF
                 AAVVLFLVTGQAVSGEALIPNLLLIHAWFPALEISFGINPVSWSLACEAFFYLCFPLF
                 LFWISGIRPERLWAWAAVVFAAIWAVPVVADLLLPSSPPLIPGLEYSAIQDWFLYTFP
                 ATRSLEFILGIILARILITGRWINVGLLPAVLLFPVFFVASLFLPGVYAISSSMMILP
                 LVLIIASGATADLQQKRTFMRNRVMVWLGDVSFALYMVHFLVIVYGADLLGFSQTEDA
                 PLGLALFMIIPFLAVSLVLSWLLYRFVELPVMRNWARPASARRKPATEPEQTPSRR"
gene             4651..5775                                  (SEQ ID NO: 5)
                 /gene="megDII"
CDS              4651..5775
                 /gene="megDII"
                 /codon_start=1
                 /transl_table=11
                 /product="TDP-3-keto-6-deoxyhexose 3-aminotransaminase"
                 /translation="MTTYVWSYLLEYERERADILDAVQKVFASGSLILGQSVENFETE
                 YARYHGIAHCVGVDNGTNAVKLALESVGVGRDDEVVTVSNTAAPTVLAIDEIGARPVF
                 VDVRDEDYLMDTDLVEAAVTPRTKAIVPVHLYGQCVDMTALRELADRRGLKLVEDCAQ
                 AHGARRDGRLAGTMSDAAAFSFYPTKVLGAYGDGGAVVTNDDETARALRRLRYYGMEE
                 VYYVTRTPGHNSRLDEVQAEILRRKLTRLDAYVAGRRAVAQRYVDGLADLQDSHGLEL
                 PVVTDGNEHVFYVYVVRHPRRDEIIKRLRDGYDISLNISYPWPVHTMTGFAHLGVASG
                 SLPVTERLAGEIFSLPMYPSLPHDLQDRVIEAVREVITGL" (SEQ ID NO: 6)
gene             5822.6595
```

FIG. 7-4

```
                /gene="megDIII"
CDS             5822..6595
                /gene="megDIII"
                /codon_start=1
                /transl_table=11
                /product="daunosaminyl-N,N-dimethyltransferase"
                /translation="MPNSHSTTSSTDVAPYERADIYHDFYHGRGKGYRAEADALVEVA
                RKHTPQAATLLDVACGTGSHLVELADSFREVVGVDLSAAMLATAARNDPGRELHQGDM
                RDFSLDRRFDVVTCMFSSTGYLVDEAELDRAVANLAGHLAPGGTLVVEPWWFPETFRP
                GWVGADLVTSGDRRISRMSHTVPAGLPDRTASRMTIHYTVGSPEAGIEHFTEVHVMTL
                FARAAYEQAFQRAGLSCSYVGHDLFSPGLFVGVAAEPGR" (SEQ ID NO: 7)
gene            6592..7197
                /gene="megDIV"
CDS             6592..7197
                /gene="megDIV"
                /codon_start=1
                /transl_table=11
                /product="TDP-4-keto-6-deoxyhexose 3,5-epimerase"
                /translation="MRVEELGIEGVFTFTPQTFADERGVFGTAYQEDVFVAALGRPLF
                PVAQVSTTRSRRGVVRGVHFTTMPGSMAKYVYCARGRAMDFAVDIRPGSPTFGRAEPV
                ELSAESMVGLYLPVGMGHLFVSLEDDTTLVYLMSAGYVPDKERAVHPLDPELALPIPA
                DLDLVMSERDRVAPTLREARDQGILPDYAACRAAAHRVVRT" (SEQ ID NO: 8)
gene            7220..8206
```

FIG. 7-5

```
                /gene="megDV"
CDS             7220..8206
                /gene="megDV"
                /codon_start=1
                /transl_table=11
                /product="TDP-4-keto-6-deoxyhexose 4-ketoreductase"
                /translation="MVVLGASGFLGSAVTHALADLPVRVRLVARREVVVPSGAVADYE
                THRVDLTEPGALAEVVADARAVFPFAAQIRGTSGWRISEDDVVAERTNVGLVRDLIAV
                LSRSPHAPVVVFPGSNTQVGRVTAGRVIDGSEQDHPEGVYDRQKHTGEQLLKEATAAG
                AIRATSLRLPPVFGVPAAGTADDRGVVSTMIRRALTGQPLTMWHDGTVRRELLYVTDA
                ARAFVTALDHADALAGRHFLLGTGRSWPLGEVFQAVSRSVARHTGEDPVPVVSVPPPA
                HMDPSDLRSVEVDPARFTAVTGWRATVTMAEAVDRTVAALAPRRAAAPSEPS"
gene            complement (8228..9220)                       (SEQ ID NO: 9)
                /gene="megDVII"
CDS             complement (8228..9220)
                /gene="megDVII"
                /codon_start=1
                /transl_table=11
                /product="TDP-4-keto-6-deoxyhexose 2,3-reductase"
                /translation="MGTTGAGSARVRVGRSALHTSRLWLGTVNFSGRVTDDDALRLMD
                HALERGVNCIDTADIYGWRLYKGHTEELVGRWFAQGGGRREETVLATKVGSEMSERVN
                DGGLSARHIVAACENSLRRLGVDHIDIYQTHHIDRAAPWDEVWQAAEHLVGSGKVGYV
                GSSNLAGWHIAAAQESAARRNLLGMISHQCLYNLAVRHPELDVLPAAQAYGVGVFAWS
```

FIG. 7-6

```
                PLHGGLLSGVLEKLAAGTAVKSAQGRAQVLLPAVRPLVEAYEDYCRRLGADPAEVGLA
                WVLSRPGILGAVIGPRTPEQLDSALRAAELTLGEEELRELEAIFPAPAVDGPVP"
     gene       complement (9226..10479)                    (SEQ ID NO: 10)
                /gene="megBV"
     CDS        complement (9226..10479)
                /gene="megBV"
                /codon_start=1
                /transl_table=11
                /product="TDP-mycarose glycosyltransferase"
                /translation="MRVLLTSFAHRTHFQGLVPLAWALHTAGHDVRVASQPELTDVVV
                GAGLTSVPLGSDHRLFDISPEAAAQVHRYTTDLDFARRGPELRSWEFLHGIEEATSRF
                VFPVVNNDSFVDELVEFAMDWRPDLVLWEPFTFAGAVAAKACGAAHARLLWGSDLTGY
                FRSRSQDLRGQRPADDRPDPLGGWLTEVAGRFGLDYSEDLAVGQWSVDQLPESFRLET
                GLESVHTRTLPYNGSSVVPQWLRTSDGVRRVCFTGGYSALGITSNPQEFLRTLATLAR
                FDGEIVVTRSGLDPASVPDNVRLVDFVPMNILLPGCAAVIHHGGAGSWATALHHGVPQ
                ISVAHEWDCVLRGQRTAELGAGVFLRPDEVDADTLWQALATVVEDRSHAENAEKLRQE
                ALAAPTPAEVVPVLEALAHQHRADR" (SEQ ID NO: 11)
     gene       complement (10483..11424)
                /gene="megBIV"
     CDS        complement (10483..11424)
                /gene="megBIV"
                /codon_start=1
                /transl_table=11
```

FIG. 7-7

```
            /product="TDP-4-keto-6-deoxyhexose 4-ketoreductase"
            /translation="MTRHVTLLGVSGFVGSALLREFTTHPLRLRAVARTGSRDQPPGS
            AGIEHLRVDLLEPGRVAQVVADTDVVVHLVAYAAGGSTWRSAATVPEAERVNAGIMRD
            LVAALRARPGPAPVLLFASTTQAANPAAPSRYAQHKIEAERILRQATEDGVVDGVILR
            LPAIYGHSGPSGQTGRGVVTAMIRRALAGEPITMWHEGSVRRNLLHVEDVATAFTAAL
            HNHEALVGDVWTPSADEARPLGEIFETVAASVARQTGNPAVPVVSVPPPENAEANDFR
            SDDFDSTEFRTLTGWHPRVPLAEGIDRTVAALISTKE"      (SEQ ID NO: 12)
gene        12181..22821
            /gene="megAI"
CDS         12181..22821
            /gene="megAI"
            /note="polyketide synthase"
            /codon_start=1
            /transl_table=11
            /product="megalomicin 6-deoxyerythronolide B synthase 1"
            /translation="MVDVPDLLGTRTPHPGPLPFPWPLCGHNEPELRARARQLHAYLE
            GISEDDVVAVGAALARETRAQDGPHRAVVVASSVTELTAALAALAQGRPHPSVVRGVA
            RPTAPVVFVLPGQGAQWPGMATRLLAESPVFAAAMRACERAFDEVTDWSLTEVLDSPE
            HLRRVEVVQPALFAVQTSLAALWRSFGVRPDAVLGHSIGELAAAEVCGAVDVEAAARA
            AALWSREMVPLVGRGDMAAVALSPAELAARVERWDDDVVPAGVNGPRSVLLTGAPEPI
            ARRVAELAAQGVRAQVVNVSMAAHSAQVDAVAEGMRSALTWFAPGDSDVPYYAGLTGG
            RLDTRELGADHWPRSFRLPVRFDEATRAVLELQPGTFIESSPHPVLAASLQQTLDEVG
            SPAAIVPTLQRDQGGLRRFLLAVAQAYTGGVTVDWTAAYPGVTPGHLPSAVAVETDEG
```

FIG. 7-8

PSTEFDWAAPDHVLRARLLEIVGAETAALAGREVDARATFRELGLDSVLAVQLRTRLA
TATGRDLHIAMLYDHPTPHALTEALLRGPQEEPGRGEETAHPTEAEPDEPVAVVAMAC
RLPGGVTSPEEFWELLAEGRDAVGGLPTDRGWDLDSLFHPDPTRSGTAHQRAGGFLTG
ATSFDAAFFGLSPREALAVEPQQRITLELSWEVLERAGIPPTSLRTSRTGVFVGLIPQ
EYGPRLAEGGEGVEGYLMTGTTTSVASGRVAYTLGLEGPAISVDTACSSSLVAVHLAC
QSLRRGESTMALAGGVTVMPTPGMLVDFSRMNSLAPDGRSKAFSAAADGFGMAEGAGM
LLLERLSDARRHGHPVLAVIRGTAVNSDGASNGLSAPNGRAQVRVIRQALAESGLTPH
TVDVVETHGTGTRLGDPIEARALSDAYGGDREHPLRIGSVKSNIGHTQAAAGVAGLIK
LVLAMQAGVLPRTLHADEPSPEIDWSSGAISLLQEPAAWPAGERPRRAGVSSFGISGT
NAHAIIEEAPPTGDDTRPDRMGPVVPWVLSASTGEALRARAARLAGHLREHPDQDLDD
VAYSLATGRAALAYRSGFVPADASTALRILDELAAGGSGDAVTGTARAPQRVVFVFPG
QGWQWAGMAVDLLDGDPVFASVLRECADALEPYLDFEIVPFLRAEAQRRTPDHTLSTD
RVDVVQPVLFAVMVSLAARWRAYGVEPAAVIGHSQGEIAAACVAGALSLDDAARAVAL
RSRVIATMPGNGAMASIAASVDEVAARIDGRVEIAAVNGPRAVVVSGDRDDLDRLVAS
CTVEGVRAKRLPVDYASHSSHVEAVRDALHAELGEFRPLPGFVPFYSTVTGRWVEPAE
LDAGYWFRNLRHRVRFADAVRSLADQGYTTFLEVSAHPVLTTAIEEIGEDRGGDLVAV
HSLRRGAGGPVDFGSALARAFVAGVAVDWESAYQGAGARRVPLPTYPFQRERFWLEPN
PARRVADSDDVSSLRYRIEWHPTDPGEPGRLDGTWLLATYPGRADDRVEAARQALESA
GARVEDLVVEPRTGRVDLVRRLDAVGPVAGVLCLFAVAEPAAEHSPLAVTSLSDTLDL
TQAVAGSGRECPIWVVTENAVAVGPFERLRDPAHGALWALGRVVALENPAVWGGLVDV
PSGSVAELSRHLGTTLSGAGEDQVALRPDGTYARRWCRAGAGGTGRWQPRGTVLVTGG
TGGVGRHVARWLARQGTPCLVLASRRGPDADGVEELLTELADLGTRATVTACDVTDRE
QLRALLATVDDEHPLSAVFHVAATLDDGTVETLTGDRIERANRAKVLGARNLHELTRD

FIG. 7-9

ADLDAFVLFSSSTAAFGAPGLGGYVPGNAYLDGLAQQRRSEGLPATSVAWGTWAGSGM
AEGPVADRFRRHGVMEMHPDQAVEGLRVALVQGEVAPIVVDIRWDRFLLAYTAQRPTR
LFDTLDEARRAAPGPDAGPGVAALAGLPVGEREKAVLDLVRTHAAAVLGHASAEQVPV
DRAFAELGVDSLSALELRNRLTTATGVRLATTTVFDHPDVRTLAGHLAAELGGGSGRE
RPGGEAPTVAPTDEPIAIVGMACRLPGGVDSPEQLWELIVSGRDTASAAPGDRSWDPA
ELMVSDTTGTRTAFGNFMPGAGEFDAAFFGISPREALAMDPQQRHALETTWEALENAG
IRPESLRGTDTGVFVGMSHQGYATGRPKPEDEVDGYLLTGNTASVASGRIAYVLGLEG
PAITVDTACSSSLVALHVAAGSLRSGDCGLAVAGGVSVMAGPEVFREFSRQGALAPDG
RCKPFSDEADGFGLGEGSAFVVLQRLSVAVREGRRVLGVVVGSAVNQDGASNGLAAPS
GVAQQRVIRRAWGRAGVSGGDVGVVEAHGTGTRLGDPVELGALLGTYGVGRGGVGPVV
VGSVKANVGHVQAAAGVVGVIKVVLGLGRGLVGPMVCRGGLSGLVDWSSGGLVVADGV
RGWPVGVDGVRRGGVSAFGVSGTNAHVVVAEAPGSVVGAERPVEGSSRGLVGVVGGVV
PVVLSAKTETALHAQARRLADHLETHPDVPMTDVVWTLTQARQRFDRRAVLLAADRTQ
AVERLRGLAGGEPGTGVVSGVASGGGVVFVFPGQGGQWVGMARGLLSVPVFVESVVEC
DAVVSSVVGFSVLGVLEGRSGAPSLDRVDVVQPVLFVVMVSLARLWRWCGVVPAAVVG
HSQGEIAAAVVAGVLSVGDGARVVALRARALRALAGHGGMASVRRGRDDVQKLLDSGP
WTGKLEIAAVNGPDAVVVSGDPRAVTELVEHCDGIGVRARTIPVDYASHSAQVESLRE
ELLSVLAGIEGRPATVPFYSTLTGGFVDGTELDADYWYRNLRHPVRFHAAVEALAARD
LTTFVEVSPHPVLSMAVGETLADVESAVTVGTLERDTDDVERFLTSLAEAHVHGVPVD
WAAVLGSGTLVDLPTYPFQGRRFWLHPDRGPRDDVADWFHRVDWTATATDGSARLDGR
WLVVVPEGYTDDGWVVEVRAALAAGGAEPVVTTVEEVTDRVGDSDAVVSMLGLADDGA
AETLALLRRLDAQASTTPLWVVTVGAVAPAGPVQRPEQATVWGLALVASLERGHRWTG
LLDLPQTPDPQLRPRLVEALAGAEDQVAVRADAVHARRIVPTPVTGAGPYTAPGGTIL

FIG. 7-10

```
                VTGGTAGLGAVTARWLAERGAEHLALVSRRGPGTAGVDEVVRDLTGLGVRVSVHSCDV
                GDRESVGALVQELTAAGDVVRGVVHAAGLPQQVPLTDMDPADLADVVAVKVDGAVHLA
                DLCPEAELFLLFSSGAGVWGSARQGAYAAGNAFLDAFARHRRDRGLPATSVAWGLWAA
                GGMTGDQEAVSFLRERGVRPMSVPRALSALERVLTAGETAVVVADVDWAAFAESYTSA
                RPRPLLHRLVTPAAAVGERDEPREQTLRDRLAALPRAERSAELVRLVRRDAAAVLGSD
                AKAVPATTPFKDLGFDSLAAVRFRNRLAAHTGLRLPATLVFEHPNAAAVADLLHDRLG
                EAGEPTPVRSVGAGLAALEQALPDASDTERVELVERLERMLAGLRPEAGAGADAPTAG
                DDLGEAGVDELLDALERELDAR" (SEQ ID NO: 13)
misc_feature    12505..13470
                /gene="megAI"
                /function="AT-L"
misc_feature    13576..13791
                /gene="megAI"
                /function="ACP-L"
misc_feature    13849..15126
                /gene="megAI"
                /function="KS1"
misc_feature    15427..16476
                /gene="megAI"
                /function="AT1"
misc_feature    17155..17694
                /gene="megAI"
                /function="KR1"
```

FIG. 7-11

```
misc_feature    17947..18207
                /gene="megAI"
                /function="ACP1"
misc_feature    18268..19548
                /gene="megAI"
                /function="KS2"
misc_feature    19876..20910
                /gene="megAI"
                /function="AT2"
misc_feature    21517..22053
                /gene="megAI"
                /function="KR2"
misc_feature    22318..22575
                /gene="megAI"
                /function="ACP2"
gene            22867..33555
                /gene="megAII"
CDS             22867..33555
                /gene="megAII"
                /note="polyketide synthase"
                /codon_start=1
                /transl_table=11
                /product="megalomicin 6-deoxyerythronolide B synthase 2"
```

FIG. 7-12

/translation="MTDNDKVAEYLRRATLDLRAARKRLRELQSDPIAVVGMACRLPG
GVHLPQHLWDLLRQGHETVSTFPTGRGWDLAGLFHPDPDHPGTSYVDRGGFLDDVAGF
DAEFFGISPREATAMDPQQRLLLETSWELVESAGIDPHSLRGTPTGVFLGVARLGYGE
NGTEAGDAEGYSVTGVAPAVASGRISYALGLEGPSISVDTACSSSLVALHLAVESLRL
GESSLAVVGGAAVMATPGVFVDFSRQRALAADGRSKAFGAAADGFGFSEGVSLVLLER
LSEAESNGHEVLAVIRGSALNQDGASNGLAAPNGTAQRKVIRQALRNCGLTPADVDAV
EAHGTGTTLGDPIEANALLDTYGRDRDPDHPLWLGSVKSNIGHTQAAAGVTGLLKMVL
ALRHEELPATLHVDEPTPHVDWSSGAVRLATRGRPWRRGDRPRRAGVSAFGISGTNAH
VIVEEAPERTTERTVGGDVGPVPLVVSARSAAALRAQAAQVAELVEGSDVGLAEVGRS
LAVTRARHEHRAAVVASTRAEAVRGLREVAAVEPRGEDTVTGVAETSGRTVVFLFPGQ
GSQWVGMGAELLDSAPAFADTIRACDEAMAPLQDWSVSDVLRQEPGAPGLDRVDVVQP
VLFAVMVSLARLWQSYGVTPAAVVGHSQGEIAAAHVAGALSLADAARLVVGRSRLLRS
LSGGGGMSAVALGEAEVRRRLRSWEDRISVAAVNGPRSVVVAGEPEALREWGREREAE
GVRVREIDVDYASHSPQIDRVRDELLTVTGEIEPRSAEITFYSTVDVRAVDGTDLDAG
YWYRNLRETVRFADAMTRLADSGYDAFVEVSPHPVVVSAVAEAVEEAGVEDAVVVGTL
SRGDGGPGAFLRSAATAHCAGVDVDWTPALPGAATIPLPTYPFQRKPYWLRSSAPAPA
SHDLAYRVSWTPITPPGDGVLDGDWLVVHPGGSTGWVDGLAAAITAGGGRVVAHPVDS
VTSRTGLAEALARRDGTFRGVLSWVATDERHVEAGAVALLTLAQALGDAGIDAPLWCL
TQEAVRTPVDGDLARPAQAALHGFAQVARLELARRFGGVLDLPATVDAAGTRLVAAVL
AGGGEDVVAVRGDRLYGRRLVRATLPPPGGGFTPHGTVLVTGAAGPVGGRLARWLAER
GATRLVLPGAHPGEELLTAIRAAGATAVVCEPEAEALRTAIGGELPTALVHAETLTNF
AGVADADPEDFAATVAAKTALPTVLAEVLGDHRLEREVYCSSVAGVWGGVGMAAYAAG
SAYLDALVEHRRARGHASASVAWTPWALPGAVDDGRLRERGLRSLDVADALGTWERLL

FIG. 7-13

RAGAVSVAVADVDWSVFTEGFAAIRPTPLFDELLDRRGDPDGAPVDRPGEPAGEWGRR
IAALSPQEQRETLLTLVGETVAEVLGHETGTEINTRRAFSELGLDSLGSMALRQRLAA
RTGLRMPASLVFDHPTVTALARYLRRLVVGDSDPTPVRVFGPTDEAEPVAVVGIGCRF
PGGIATPEDLWRVVSEGTSITTGFPTDRGWDLRRLYHPDPDHPGTSYVDRGGFLDGAP
DFDPGFFGITPREALAMDPQQRLTLEIAWEAVERAGIDPETLLGSDTGVFVGMNGQSY
LQLLTGEGDRLNGYQGLGNSASVLSGRVAYTFGWEGPALTVDTACSSSLVAIHLAMQS
LRRGECSLALAGGVTVMADPYTFVDFSAQRGLAADGRCKAFSAQASGFALAEGVAALV
LEPLSKARRNGHQVLAVLRGSAVNQDGASNGLAAPNGPSQERVIRQALTASGLRPADV
DMVEAHGTGTELGDPIEAGALIAAYGRDRDRPLWLGSVKTNIGHTQAAAGAAGVIKAV
LAMRHGVLPRSLHADELSPHIDWADGKVEVLREARQWPPGERPRRAGVSSFGVSGTNA
HVIVEEAPAEPDPEPVPAAPGGPLPFVLHGRSVQTVRSQARTLAEHLRTTGHRDLADT
ARTLATGRARFDVRAAVLGTDREGVCAALDALAQDRPSPDVVAPAVFAARTPVLVFPG
QGSQWVGMARDLLDSSEVFAESMGRCAEALSPYTDWDLLDVVRGVGDPDPYDRVDVLQ
PVLFAVMVSLARLWQSYGVTPGAVVGHSQGEIAAAHVAGALSLADAARVVALRSRVLR
ELDDQGGMVSVGTSRAELDSVLRRWDGRVAVAAVNGPGTLVVAGPTAELDEFLAVAEA
REMRPRRIAVRYASHSPEVARVEQRLAAELGTVTAVGGTVPLYSTATGDLLDTTAMDA
GYWYRNLRQPVLFEHAVRSLLERGFETFIEVSPHPVLLMAVEETAEDAERPVTGVPTL
RRDHDGPSEFLRNLLGAHVHGVDVDLRPAVAHGRLVDLPTYPFDRQRLWPKPHRRADT
SSLGVRDSTHPLLHAAVDVPGHGGAVFTGRLSPDEQQWLTQHVVGGRNLVPGSVLVDL
ALTAGADVGVPVLEELVLQQPLVLTAAGALLRLSVGAADEDGRRPVEIHAAEDVSDPA
EARWSAYATGTLAVGVAGGGRDGTQWPPPGATALTLTDHYDTLAELGYEYGPAFQALR

FIG. 7-14

```
               AAWQHGDVVYAEVSLDAVEEGYAFDPVLLDAVAQTFGLTSRAPGKLPFAWRGVTLHAT
               GATAVRVVATPAGPDAVALRVTDPTGQLVATVDALVVRDAGADRDQPRGRDGDLHRLE
               WVRLATPDPTPAAVVHVAADGLDDLLRAGGPAPQAVVVRYRPDGDDPTAEARHGVLWA
               ATLVRRWLDDDRWPATTLVVATSAGVEVSPGDDVPRPGAAAVWGVLRCAQAESPDRFV
               LVDGDPETPPAVPDNPQLAVRDGAVFVPRLTPLAGPVPAVADRAYRLVPGNGGSIEAV
               AFAPVPDADRPLAPEEVRVAVRATGVNFRDVLLALGMYPEPAEMGTEASGVVTEVGSG
               VRRFTPGQAVTGLFQGAFGPVAVADHRLLTPVPDGWRAVDAAAVPIAFTTAHYALHDL
               AGLQAGQSVLVHAAAGGVGMAAVALARRAGAEVFATASPAKHPTLRALGLDDDHIASS
               RESGFGERPAARTGGRGVDVVLNSLTGDLLDESARLLADGGVFVEMGKTDLRPAEQFR
               GRYVPFDLAEAGPDRLGEILEEVVGLLAAGALDRLPVSVWELSAAPAALTHMSRGRHV
               GKLVLTQPAPVHPDGTVLVTGGTGTLGRLVARHLVTGHGVPHLLVASRRGPAAPGAAE
               LRADVEGLGATIEIVACDTADREALAALLDSIPADRPLTGVVHTAGVLADGLVTSIDG
               TATDQVLRAKVDAAWHLHDLTRDADLSFFVLFSSAASVLAGPGQGVYAAANGVLNALA
               GQRRALGLPAKALGWGLWAQASEMTSGLGDRIARTGVAALPTERALALFDAALRSGGE
               VLFPLSVDRSALRRAEYVPEVLRGAVRSTPRAANRAETPGRGLLDRLVGAPETDQVAA
               LAELVRSHAAAVAGYDSADQLPERKAFKDLGFDSLAAVELRNRLGVTTGVRLPSTLVF
               DHPTPLAVAEHLRSELFADSAPDVGVGARLDDLERALDALPDAQGHADVGARLEALLR
               RWQSRRPPETEPVTISDDASDDELFSMLDRRLGGGGDV" (SEQ ID NO: 14)
misc_feature   22957..24237
               /gene="megAII"
               /function="KS3"
misc_feature   24544..25581
               /gene="megAII"
               /function="AT3"
```

FIG. 7-15

```
misc_feature  26230..26733
              /gene="megAII"
              /function="KR3" (inactive)
misc_feature  26998..27258
              /gene="megAII"
              /function="ACP3"
misc_feature  27393..28590
              /gene="megAII"
              /function="KS4"
misc_feature  28897..29931
              /gene="megAII"
              /function="AT4"
misc_feature  29953..30477
              /gene="megAII"
              /function="DH4"
misc_feature  31396..32244
              /gene="megAII"
              /function="ER4"
misc_feature  32257..32799
              /gene="megAII"
              /function="KR4"
misc_feature  33052..33312
              /gene="megAII"
              /function="ACP4"
```

FIG. 7-16

```
gene            33666..43271
                /gene="megAIII"
CDS             33666..43271
                /gene="megAIII"
                /note="polyketide synthase"
                /codon_start=1
                /transl_table=11
                /product="megalomicin 6-deoxyerythronolide B synthase 3"
                /translation="MSESSGMTEDRLRRYLKRTVAELDSVTGRLDEVEYRAREPIAVV
                GMACRFPGGVDSPEAFWEFIRDGGDAIAEAPTDRGWPPAPRPRLGGLLAEPGAFDAAF
                FGISPREALATDPQQRLMLEISWEALERAGFDPSSLRGSAGGVFTGVGAVDYGPRPDE
                APEEVLGYVGIGTASSVASGRVAYTLGLEGPAVTVDTACSSGLTAVHLAMESLRRDEC
                TLVLAGGVTVMSSPGAFTEFRSQGGLAEDGRCKPFSRAADGFGLAEGAGVLVLQRLSV
                ARAEGRPVLAVLRGSAINQDGASNGLTAPSGPAQRRVIRQALERARLRPVDVDYVEAH
                GTGTRLGDPIEAHALLDTYGADREPGRPLWVGSVKSNIGHTQAAAGVAGVMKTVLALR
                HREIPATLHFDEPSPHVDWDRGAVSVVSETRPWPVGERPRRAGVSSFGISGTNAHVIV
                EEAPSPQAADLDPTPGPATGATPGTDAAPTAEPGAEAVALVFSARDERALRAQAARLA
                DRLTDDPAPSLRDTAFTLVTRRATWEHRAVVVGGGEEVLAGLRAVAGGRPVDGAVSGR
                ARAGRRVVLVFPGQGAQWQGMARDLLRQSPTFAESIDACERALAPHVDWSLREVLDGE
                QSLDPVDVVQPVLFAVMVSLARLWQSYGVTPGAVVGHSQGEIAAAHVAGALSLADAAR
                VVALRSRVLRRLGGHGGMASFGLHPDQAAERIARFAGALTVASVNGPRSVVLAGENGP
                LDELIAECEAEGVTARRIPVDYASHSPQVESLREELLAALAGVRPVSAGIPLYSTLTG
                QVIETATMDADYWFANLREPVRFQDATRQLAEAGFDAFVEVSPHPVLTVGVEATLEAV
                LPPDADPCVTGTLRRERGGLAQFHTALAEAYTRGVEVDWRTAVGEGRPVDLPVYPFQR
```

FIG. 7-17

QNFWLPVPLGRVPDTGDEWRYQLAWHPVDLGRSSLAGRVLVVTGAAVPPAWTDVVRDG
LEQRGATVVLCTAQSRARIGAALDAVDGTALSTVVSLLALAEGGAVDDPSLDTLALVQ
ALGAAGIDVPLWLVTRDAAAVTVGDDVDPAQAMVGGLGRVVGVESPARWGGLVDLREA
DADSARSLAAILADPRGEEQFAIRPDGVTVARLVPAPARAAGTRWTPRGTVLVTGGTG
GIGAHLARWLAGAGAEHLVLLNRRGAEAAGAADLRDELVALGTGVTITACDVADRDRL
AAVLDAARAQGRVVTAVFHAAGISRSTAVQELTESEFTEITDAKVRGTANLAELCPEL
DALVLFSSNAAVWGSPGLASYAAGNAFLDAFARRGRRSGLPVTSIAWGLWAGQNMAGT
EGGDYLRSQGLRAMDPQRAIEELRTTLDAGDPWVSVVDLDRERFVELFTAARRRPLFD
ELGGVRAGAEETGQESDLARRLASMPEAERHEHVARLVRAEVAAVLGHGTPTVIERDV
AFRDLGFDSMTAVDLRNRLAAVTGVRVATTIVFDHPTVDRLTAHYLERLVGEPEATTP
AAAVVPQAPGEADEPIAIVGMACRLAGGVRTPDQLWDFIVADGDAVTEMPSDRSWDLD
ALFDPDPERHGTSYSRHGAFLDGAADFDAAFFGISPREALAMDPQQRQVLETTWELFE
NAGIDPHSLRGTDTGVFLGAAYQGYGQNAQVPKESEGYLLTGGSSAVASGRIAYVLGL
EGPAITVDTACSSSLVALHVAAGSLRSGDCGLAVAGGVSVMAGPEVFTEFSRQGALAP
DGRCKPFSDQADGFGFAEGVAVVLLQRLSVAVREGRRVLGVVVGSAVNQDGASNGLAA
PSGVAQQRVIRRAWGRAGVSGGDVGVVEAHGTGTRLGDPVELGALLGTYGVGRGGVGP
VVVGSVKANVGHVQAAAGVVGVIKVVLGLGRGLVGPMVCRGGLSGLVDWSSGGLVVAD
GVRGWPVGVDGVRRGGVSAFGVSGTNAHVVVAEAPGSVVGAERPVEGSSRGLVGVAGG
VVPVVLSAKTETALTELARRLHDAVDDTVALPAVAATLATGRAHLPYRAALLARDHDE
LRDRLRAFTTGSAAPGVVSGVASGGGVVFVFPGQGGQWVGMARGLLSVPVFVESVVEC
DAVVSSVVGFSVLGVLEGRSGAPSLDRVDVVQPVLFVVMVSLARLWRWCGVVPAAVVG
HSQGEIAAAVVAGVLSVGDGARVVALRARALRALAGHGGMVSLAVSAERARELIAPWS
DRISVAAVNSPTSVVVSGDPQALAALVAHCAETGERAKTLPVDYASHSAHVEQIRDTI
LTDLADVTARRPDVALYSTLHGARGAGTDMDARYWYDNLRSPVRFDEAVEAAVADGYR

FIG. 7-18

```
                VFVEMSPHPVLTAAVQEIDDETVAIGSLHRDTGERHLVAELARAHVHGVPVDWRAILP
                ATHPVPLPNYPFEATRYWLAPTAADQVADHRYRVDWRPLATTPAELSGSYLVFGDAPE
                TLGHSVEKAGGLLVPVAAPDRESLAVALDEAAGRLAGVLSFAADTATHLARHRLLGEA
                DVEAPLWLVTSGGVALDDHDPIDCDQAMVWGIGRVMGLETPHRWGGLVDVTVEPTAED
                GVVFAALLAADDHEDQVALRDGIRHGRRLVRAPLTTRNARWTPAGTALVTGGTGALGG
                HVARYLARSGVTDLVLLSRSGPDAPGAAELAAELADLGAEPRVEACDVTDGPRLRALV
                QELREQDRPVRIVVHTAGVPDSRPLDRIDELESVSAAKVTGARLLDELCPDADTFVLF
                SSGAGVWGSANLGAYAAANAYLDALAHRRRQAGRAATSVAWGAWAGDGMATGDLDGLT
                RRGLRAMAPDRALRACTRRWTTHDTCVSVADVDWDRFAVGFTAARPRPLIDELVTSAP
                VAAPTAAAAPVPAMTADQLLQFTRSHVAAILGHQDPDAVGLDQPFTELGFDSLTAVGL
                RNQLQQATGRTLPAALVFQHPTVRRLADHLAQQLDVGTAPVEATGSVLRDGYRRAGQT
                GDVRSYLDLLANLSEFRERFTDAASLGGQLELVDLADGSGPVTVICCAGTAALSGPHE
                FARLASALRGTVPVRALAQPGYEAGEPVPASMEAVLGVQADAVLAAQGDTPFVLVGHS
                AGALMAYALATELADRGHPPRGVVLLDVYPPGHQEAVHAWLGELTAALFDHETVRMDD
                TRLTALGAYDRLTGRWRPRDTGLPTLVVAASEPMGEWPDDGWQSTWPFGHDRVTVPGD
                HFSMVQEHADAIARHIDAWLSGERA" (SEQ ID NO: 15)
misc_feature    33780..35027
                /gene="megAIII"
                /function="KS5"
misc_feature    35385..36419
                /gene="megAIII"
                /function="AT5"
```

FIG. 7-19

```
misc_feature  37068..37604
              /gene="megAIII"
              /function="KR5"
misc_feature  37860..38120
              /gene="megAIII"
              /function="ACP5"
misc_feature  38187..39470
              /gene="megAIII"
              /function="KS6"
misc_feature  39795..40811
              /gene="megAIII"
              /function="AT6"
misc_feature  41406..41936
              /gene="megAIII"
              /function="KR6"
misc_feature  42168..42425
              /gene="megAIII"
              /function="ACP6"
misc_feature  42585..43271
              /gene="megAIII"
              /function="TE"
gene          43268..44344
              /gene="megCII"
```

FIG. 7-20

```
CDS             43268..44344
                /gene="megCII"
                /codon_start=1
                /transl_table=11
                /product="TDP-4-keto-6-deoxyglucose 3,4-isomerase"
                /translation="MNTTDRAVLGRRLQMIRGLYWGYGSNGDPYPMLLCGHDDDPHRW
                YRGLGGSGVRRSRTETWVVTDHATAVRVLDDPTFTRATGRTPEWMRAAGAPASTWAQP
                FRDVHAASWDAELPDPQEVEDRLTGLLPAPGTRLDLVRDLAWPMASRGVGADDPDVLR
                AAWDARVGLDAQLTPQPLAVTEAAIAAVPGDPHRRALFTAVEMTATAFVDAVLAVTAT
                AGAAQRLADDPDVAARLVAEVLRLHPTAHLERRTAGTETVVGEHTVAAGDEVVVVVAA
                ANRDAGVFADPDRLDPDRADADRALSAQRGHPGRLEELVVVLTTAALRSVAKALPGLT
                AGGPVVRRRRSPVLRATAHCPVEL" (SEQ ID NO: 16)
gene            44355..45623
                /gene="megCIII"
CDS             44355..45623
                /gene="megCIII"
                /codon_start=1
                /transl_table=11
                /product="TDP-desosamine glycosyltransferase"
                /translation="MRVVFSSMASKSHLFGLVPLAWAFRAAGHEVRVVASPALTDDIT
                AAGLTAVPVGTDVDLVDFMTHAGYDIIDYVRSLDFSERDPATSTWDHLLGMQTVLTPT
                FYALMSPDSLVEGMISFCRSWRPDWSSGPQTFAASIAATVTGVAHARLLWGPDITVRA
                RQKFLGLLPGQPAAHREDPLAEWLTWSVERFGGRVPQDVEELVVGQWTIDPAPVGMRL
```

FIG. 7-21

```
                DTGLRTVGMRYVDYNGPSVVPDWLHDEPTRRRVCLTLGISSRENSIGQVSVDDLLGAL
                GDVDAEIIATVDEQQLEGVAHVPANIRTVGFVPMHALLPTCAATVHHGGPGSWHTAAI
                HGVPQVILPDGWDTGVRAQRTEDQGAGIALPVPELTSDQLREAVRRVLDDPAFTAGAA
                RMRADMLAEPSPAEVVDVCAGLVGERTAVG" (SEQ ID NO: 17)
gene            45620..46591
                /gene="megBII"
CDS             45620..46591
                /gene="megBII"
                /codon_start=1
                /transl_table=11
                /product="TDP-4-keto-6-deoxyglucose 2,3 dehydratase"
                /translation="MSTDATHVRLGRCALLTSRLWLGTAALAGQDDADAVRLLDHARS
                RGVNCLDTADDDSASTSAQVAEESVGRWLAGDTGRREETVLSVTVGVPPGGQVGGGGL
                SARQIIASCEGSLRRLGVDHVDVLHLPRVDRVEPWDEVWQAVDALVAAGKVCYVGSSG
                FPGWHIVAAQEHAVRRHRLGLVSHQCRYDLTSRHPELEVLPAAQAYGLGVFARPTRLG
                GLLGGDGPGAAAARASGQPTALRSAVEAYEVFCRDLGEHPAEVALAWVLSRPGVAGAV
                VGARTPGRLDSALRACGVALGATELTALDGIFPGVAAAGAAPEAWLR"
                (SEQ ID NO: 18)
gene            complement (46660..47403)
                /gene="megH"
CDS             complement (46660..47403)
                /gene="megH"
                /note="putative thioesterase"
```

FIG. 7-22

```
                  /codon_start=1
                  /transl_table=11
                  /product="TEII"
                  /translation="MNTWLRRFGSADGHRARLYCFPHAGAAADSYLDLARALAPEVDV
                  WAVQYPGRQDRRDERALGTAGEIADEVAAVLRDLVGEVPFALFGHSMGALVAYETARR
                  LEARPGVRPLRLFVSGQTAPRVHERRTDLPDEDGLVEQMRRLGVSEAALADQGLLDMS
                  LPVLRADHRVLRSYAWQAGPPLRAGITTLCGDTDPLTTVEDAQRWLPYSVVPGRTRTF
                  PGGHFYLADHVGEVAESVAPDLLRLTPTG" (SEQ ID NO: 19)
gene              complement (47411..>47981)
                  /gene="megF"
CDS               complement (47411..>47980)
                  /gene="megF"
                  /codon_start=1
                  /transl_table=11
                  /product="C-6 hydroxylase"
                  /translation="IRVQDDDADRLSRDELTSIALVLLLAGFEASVSLIGIGTYLLLT
                  HPDQLALVRKDPALLPGAVEEILRYQAPPETTTRFATAEVEIGGVTIPAYSTVLIANG
                  AANRDPGQFPDPDRFDVTRDSRGHLTFGHGIHYCMGRPLAKLEGEVALGALFDRFPKL
                  SLGFPSDEVVWRRSLLLRGIDHLPVRPNG" (SEQ ID NO: 20)
BASE COUNT         5962 a  16875 c  18045 g   7099 t
ORIGIN
        1 ctcgagccga tgctcggcgg cgcggtgggc caaccagtcg tggacgtcgt cggtggcggt
       61 gggaggtccg ccgtgccgag tcaggaaacg tattgccgat tgtgtggatt ccggagtcgc
```

FIG. 7-23

```
121  atgaccgttg acccgatccc ccatacgcct ctcccgtgat gtcgtgggcg gtccgtgcgg
181  taccgcccgg actgacattc gtcgatcaag accccgccca gtgtagggct ccgcccgcga
241  cgggagaagg tccgtcgaac aacttccggg tgaccggtcg ccggcgtcgg tgaaacgggc
301  gtcggagcac ccgatcattg ctgtcggtga acttcctaac tgtcggcgcg cacatctttc
361  tgaccggtgt gttccgtggt atgacgcgtt cccggcccgt ctggaactgt gcgtgggact
421  gaccggttgc ggcgtgtttt cgcccgtttc cgaactgcgg attcgtcgat cgcgcaggtg
481  ggagcgggtg gctgaccggg atgatctgca atcatggcgc tcaatgacga tctcttgtag
541  catggtccgc gccgagggtc cgacaggccc gaaacgcccg gcatccagcc tgttcgacga
601  cgtcgacatc accgtgcaag ccgcgatgac accgacacca cgccatgctg gtgccgcact
661  ggaagggtgg cgcgatcagg gaaatggccg tgtcactaga cagacgccaa acagctgtcc
721  gggcctgcgg aaacagcatc gatctgcgtc agccgttcat tgccccggcg gcaccgcctt
781  ggaaatccgt gccaccggtc gtccgcagtg acgatcgcgg acccgggttt cgagacagca
841  ggtagtaggc gatgcaggcg tttcgtctcg cgccggacgc gtcgcactag gtggaatccg
901  tcacagtctt caatccggga gcgttctatg gcagttggcg atcgaaggcg gctgggccgg
961  gagttgcaga tggcccgggg tctctactgg gggttcggtg ccaacggcga tctgtactcg
1021 atgctcctgt ccggacggga cgacgacccc tggacctggt acgaacggtt gcgggccgcc
1081 ggacggggac cgtacgccag tcgggccgga acgtgggtgg tcggtgacca ccggaccgcc
1141 gccgaggtgc tcgccgatcc gggcttcacc cacggcccgc ccgacgctgc ccggtggatg
1201 caggtggccc actgcccggc ggcctcctgg gccggcccct tccgggagtt ctacgcccgc
1261 accgaggacg cggcgtcggt gacagtggac gccgactggc tccagcagcg gtgcgccagg
1321 ctggtgaccg agctggggtc gcgcttcgat ctcgtgaacg acttcgcccg ggaggtcccg
1381 gtgctggcgc tcggtaccgc gcccgcactc aagggcgtgg accccgaccg tctccggtcc
```

FIG. 7-24

```
1441 tggacctcgg cgacccgggt atgcctggac gcccaggtca gcccgcaaca gctcgcggtg
1501 accgaacagg cgctgaccgc cctcgacgag atcgacgcgg tcaccggcgg tcgggacgcc
1561 gcggtgctgg tgggggtggt ggcggagctg gcggccaaca cggtgggcaa cgccgtcctg
1621 gccgtcaccg agcttcccga actggcggca cgacttgccg acgacccgga gaccgcgacc
1681 cgtgtggtga cggaggtgtc gcggacgagt cccggcgtcc acctggaacg ccgcaccgcc
1741 gcgtcggacc gccgggtggg cggggtcgac gtcccgaccg gtggcgaggt gacagtggtc
1801 gtcgccgcgg cgaaccgtga tcccgaggtc ttcaccgatc ccgaccggtt cgacgtggac
1861 cgtggcggcg acgccgagat cctgtcgtcc cggcccggct cgccccgcac cgacctcgac
1921 gccctggtgg ccaccctggc cacggcggcg ctgcgggccg ccgcgccggt gttgccccgg
1981 ctgtcccgtt ccgggccggt gatcagacga cgtcggtcac ccgtcgcccg tggtctcagc
2041 cgttgcccgg tcgagctgta gaggaagaac gatgcgcgtc gtgttttcat cgatggctgt
2101 caacagccat ctgttcgggc tggtcccgct cgcaagcgcc ttccaggcgg ccggacacga
2161 ggtacgggtc gtcgcctcgc cggccctgac cgacgacgtc accggtgccg gtctgaccgc
2221 cgtgcccgtc ggtgacgacg tggaacttgt ggagtggcac gcccacgcgg gccaggacat
2281 cgtcgagtac atgcggaccc tcgactgggt cgaccagagc cacaccacca tgtcctggga
2341 cgacctcctg ggcatgcaga ccaccttcac cccgaccttc ttcgccctga tgagccccga
2401 ctcgctcatc gacgggatgg tcgagttctg ccgctcctgg cgtcccgact ggatcgtctg
2461 ggagccgctg accttcgccg ccccgatcgc ggcccgggtc accggaaccc cgcacgcccg
2521 gatgctgtgg ggtccggacg tcgccacccg ggcccggcag agcttcctgc gactgctggc
2581 ccaccaggag gtggagcacc gggaggatcc gctggccgag tggttcgact ggacgctgcg
2641 gcgcttcggc gacgacccgc acctgagctt cgacgaggaa ctggtgctgg ggcagtggac
2701 cgtggacccc atccccgagc cgctgcggat cgacaccggc gtccggacgg tgggcatgcg
2761 gtacgtcccc tacaacggcc cctcggtggt gcccgcctgg ctgttgcggg aacccgaacg
```

FIG. 7-25

```
2821 tcggcgggtc tgcctgaccc tcggcggttc cagccgggaa cacggcatcg ggcaggtctc
2881 catcggcgag atgttggacg ccatcgccga catcgacgcc gagttcgtgg ccaccttcga
2941 cgaccagcag ttggtcggcg tgggcagcgt tccggcaaac gtccgtaccg ccgggttcgt
3001 gccgatgaac gtcctgctgc ccacctgcgc ggccaccgtg caccacggcg gcaccggcag
3061 ttggctgacc gccgccatcc acggcgtacc gcagatcatc ctctcggacg ccgacaccga
3121 ggtgcacgcc aagcagctcc aggacctcgg cgcggggctg tcgctcccgg tcgcggggat
3181 gaccgccgag cacctgcgtg gggcgatcga gcgggttctc gacgagccgg cgtaccgcct
3241 cggtgcggag cggatgcggg acgggatgcg gaccgacccg tcgccggccc aggtggtcgg
3301 catctgtcag gacctggccg ccgaccgggc ggcacgcggc aggcagccgc gtcgaaccgc
3361 cgagccgcac ctgccgcgat gacttccacc accaccggga ccggctgatg ccggtcccgg
3421 aatccacacg ccgactttcc ttctgacacg agggggcccc ggtggttacc tccaccaact
3481 tggacacgac agcacggccg gcactgaact cgttgaccgg gatgcggttc gtcgccgcct
3541 tcctggtctt cttcacgcac gtcctgtcga ggctcatccc gaacagctac gtgtacgccg
3601 acggcctgga cgccttctgg cagaccaccg gacgggtggg ggtgtcgttc ttctttattc
3661 tcagcggttt cgtgctgacc tggtcggcgc gggccagcga ctcggtgtgg tcgttctggc
3721 gcagacgggt ctgcaagctc ttccccaacc acctggtcac cgccttcgcc gccgtggtgt
3781 tgttcctggt caccgggcag gcggtgagcg gtgaggcgct gatcccgaac ctcctgctga
3841 tccacgcctg gttcccggcc ctggagatct ccttcggcat caacccggtg agctggtcgt
3901 tggcctgcga ggcgttcttc tacctgtgct tcccgctgtt cctgttctgg atctccggta
3961 tccgcccgga gcggctgtgg gcctgggccg ccgtggtgtt cgccgcgatc tgggcggtac
4021 cggtggtcgc cgacctcctg ctgccgagtt ccccgccgct gatcccgggg cttgagtact
4081 ccgccatcca ggactggttc ctctacacct tccctgcgac gcggagcctg gagttcatcc
4141 tcgggatcat cctggcccgc atcctgatca ccggtcggtg gatcaacgtc gggctgctcc
```

FIG. 7-26

```
4201 ccgcggtgct gttgttcccg gtcttcttcg tcgcctcgct cttcctgccg ggtgtctacg
4261 ccatctcctc gtcgatgatg atccttcccc tggttctgat catcgccagc ggcgcgacgg
4321 ccgacctcca gcagaagcgc accttcatgc gtaaccgggt gatggtgtgg ctcggcgacg
4381 tctccttcgc gctctacatg gtccacttcc tggtgatcgt ctacggggcg gacctgctgg
4441 ggttcagcca gaccgaggac gccccgctgg gtctcgcact cttcatgatc attccgttcc
4501 tcgcggtctc cctggtgctg tcgtggctgc tgtacaggtt cgtcgagcta cccgtcatgc
4561 gtaactgggc ccgcccggcc tccgcccggc gcaaacccgc cacggaaccc gaacagaccc
4621 cttcccgccg gtaagaagga cggtgcatcg gtgaccacct acgtctggtc ctatctgttg
4681 gagtacgaga gggaacgagc cgacatcctc gatgcggtgc agaaggtctt cgccagtggc
4741 agcctgatcc tcggtcagag tgtggagaac ttcgagaccg agtacgcccg ctaccacggg
4801 atcgcgcact gcgtgggcgt cgacaacggc accaacgctg tgaaactcgc gctggagtcg
4861 gtaggtgtcg gacgcgacga cgaggtcgtc acggtctcca acaccgccgc ccccacagtc
4921 ctggccatcg acgagatcgg cgcccggccg gtcttcgtgg acgtccgcga cgaggactac
4981 ctcatggaca ccgacctggt ggaggcggcg gtcaccccgc gtaccaaggc catcgtcccg
5041 gtgcacctgt acgggcagtg cgtggacatg acagccctgc gggaactggc cgaccggcgg
5101 ggcctcaagc tcgtggagga ctgcgcccag gcccacggtg cccggcggga cggtcggctg
5161 gccgggacga tgagcgacgc ggcggccttc tcgttctacc cgacgaaggt cctcggcgcc
5221 tacggcgacg gcggcgcggt cgtcaccaac gacgacgaga cagcccgcgc cctgcgacgg
5281 ctgcggtact acgggatgga ggaggtctac tacgtcaccc ggaccccggg tcacaacagc
5341 cgcctcgacg aggtgcaggc cgagatcctg cggcgcaaac tgacccggct cgacgcgtac
5401 gtcgcgggtc ggcgggcggt cgcccagcgg tacgtcgacg ggctcgccga cctccaagac
5461 tcgcacggcc tcgaactccc agtggtcacc gacggcaacg aacacgtctt ctacgtgtac
5521 gtcgtccgcc acccgcgccg cgacgagatc atcaagcgtc tccgggacgg gtacgacatc
```

FIG. 7-27

```
5581 tccctgaaca tcagctaccc ctggccggtg cacaccatga ccggcttcgc ccacctcggt
5641 gtcgcgtcgg ggtcgctgcc ggtcaccgaa cggctggccg gcgagatctt ctcccttccc
5701 atgtacccct ccctccctca cgacctgcag gacagggtga tcgaggcggt gcgggaggtc
5761 atcaccgggc tgtgacgagc ccgcgtgtcg tcagcgaaga cccactctgg aagggccggt
5821 catgccgaac agccactcga ccacgtcgag caccgacgtc gccccgtacg agcgggcgga
5881 catctaccac gacttctacc acggccgtgg caagggatac cgtgccgaag ccgacgcgct
5941 cgtggaggtc gcccgcaagc acaccccaca ggcggcgacc ctgctggacg tggcctgcgg
6001 gaccggatcc cacctggtcg agctggcgga cagcttccgg gaggtggtgg gggtcgacct
6061 gtcggccgcc atgctcgcca ccgccgcccg caacgacccc gggcgggaac tgcaccaggg
6121 cgacatgcgc gacttctccc tcgaccgcag gttcgacgtc gtcacctgca tgttcagctc
6181 caccggttac ctcgtcgacg aggccgaact ggaccgtgcc gtggcgaacc tggccggtca
6241 cctcgcgcct ggcggcaccc tcgtcgtgga gccctggtgg ttcccggaga cgttccggcc
6301 cggctgggtc ggggccgacc tggtcaccag cggtgaccgg aggatctccc ggatgtcgca
6361 caccgtcccg gcgggtctgc ccgaccgcac cgcctcccgg atgaccatcc actacacggt
6421 ggggtcaccg gaggccggga tcgagcactt caccgaggtg cacgtgatga ccctgttcgc
6481 ccgcgccgcc tacgagcagg ccttccagcg ggcgggcctg agctgctcgt acgtcggcca
6541 cgacctgttc tcgccgggcc ttttcgtcgg ggtcgccgcg gagccggggc ggtgagggtc
6601 gaggagctgg gcatcgaggg ggtcttcacc ttcaccccgc agacgttcgc cgacgagcgg
6661 ggggtgttcg gcacggcgta ccaggaggac gtgttcgtgg cggcgctcgg ccgcccgctg
6721 ttcccggtgg cccaggtcag caccacccgg tcccggcggg gtgtggtccg gggggtgcac
6781 ttcacgacga tgcccggctc catggcgaag tacgtctact gcgccagggg tagggcgatg
6841 gacttcgccg tcgacatccg gcccggttcc ccgaccttcg gccgggccga gccggtcgag
6901 ctctccgccg agtcgatggt cgggctgtac cttcccgtgg gcatgggcca cctgttcgtc
```

FIG. 7-28

```
6961 tccctggagg acgacaccac cctcgtctac ctgatgtccg ccggttacgt ccccgacaag
7021 gaacgggcgg tgcaccccct ggatccggag ctggcgttgc cgatcccggc cgacctcgac
7081 ctcgtcatgt ccgagcggga ccgggtcgca cccaccctcc gggaggcccg ggaccagggg
7141 atcctgcccg actacgccgc ctgccgggcc gccgcgcacc gggtggtgcg gacgtgaccc
7201 cggccgggcg tgcgggccgg tggtggtgct cggcgcgtcg ggtttcctgg gttcggcggt
7261 cacccacgcc ctggccgacc tcccggtgcg ggtgcggctc gtcgcccggc gggaggtcgt
7321 cgtgccctcc ggtgccgtcg ccgactacga gacgcaccgg gtggacctca ccgaacccgg
7381 agcgctcgcg gaggtggtcg cggacgcccg ggcggtcttc ccgttcgccg cccagatcag
7441 gggtacgtca gggtggcgga tcagcgagga cgacgtggtc gccgaacgga cgaacgtcgg
7501 cctggtccgg gacctgatcg ccgtcctgtc ccgctcgccg cacgccccgg tggtggtctt
7561 cccgggcagc aacacgcagg tcggcagggt caccgccggc cgggtcatcg acggcagcga
7621 gcaggaccac cccgagggcg tctacgacag gcagaaacac accggggaac agctgctcaa
7681 ggaggccact gcggccgggg cgatccgggc gaccagtctg cggctgcccc cggtgttcgg
7741 ggtgcccgcc gccggcaccg ccgacgaccg gggggtggtc tccaccatga tccgtcgggc
7801 cctgaccggc caaccgctga cgatgtggca cgacggcacc gtccggcgtg aactgctgta
7861 cgtgaccgac gccgcccggg ccttcgtcac cgccctggac cacgccgacg cgctcgccgg
7921 acgccacttc ctgttgggga cggggcgttc ctggccgctg ggcgaggtct tccaggcggt
7981 ctcgcgcagc gtcgcccggc acaccggcga ggacccggtg ccggtggtct cggtgccgcc
8041 tccggcgcac atggacccgt cggacctgcg cagcgtggag gtcgaccccg cccggttcac
8101 ggctgtcacc gggtggcggg ccacggtcac gatggcggag gcggtcgacc ggacggtggc
8161 ggcgttggcc ccccgccggg ccgccgcccc gtccgagccc tcctgaccgg ggtcacccgg
8221 gttcgtccta cggcaccggc ccgtcgacgg ccggtgccgg gaagatcgct tcgagttccc
8281 ggagttcctc ctcgcccagc gtcagctcgg cggcccgtaa cgccgagtcg agctgctcgg
```

FIG. 7-29

```
8341 gtgtgcgggg gccgatgaca gcgcccagga tcccggggcg ggacaggacc caggccagac
8401 cgacctcggc cgggtccgcg ccgaggcgtc ggcagtagtc ctcgtacgcc tcgacgaggg
8461 ggcgtacggc ggggaggagc acctgggcgc gtccctgcgc cgacttgacg gcggttccgg
8521 ctgccaactt ctccagtacg ccgctgagca gcccgccgtg caggggggac caggcgaaca
8581 cgcccacccc gtacgcctgg gcggcgggca ggacgtccag ctcggggtgg cggacggcca
8641 ggttgtacag gcactggtgg gagatcatgc cgagcaggtt gcggcgtgcc gcgctctcct
8701 gggcggcggc gatgtgccag cccgccaggt tggaggagcc gacgtacccg accttcccac
8761 tgccgaccag atgttcggcg gcctgccaca cctcgtccca cggtgcggcg cggtcgatgt
8821 ggtgcgtctg gtagatgtcg atgtggtcga ccccgaggcg gcggagggag ttctcgcagg
8881 cggcgacgat gtgtcgggcg gagagcccgc cgtcgttgac ccgttcgctc atctcgctgc
8941 ccaccttggt cgccaggacg gtctcctcgc gtcgacctcc gccctgggcg aaccaccgtc
9001 cgacgagttc ctcggtgtgg cccttgtaga gccgccagcc gtagatgtcg gcggtgtcga
9061 tgcagttgac gccccgctcg agggcgtggt ccatcagccg cagcgcgtcg tcgtcggtca
9121 cccgtccact gaagttcacg gtgccgagcc agagtcggct ggtgtgcaac gccgatcgtc
9181 cgacgcgtac ccgggcggac ccggccccgg tggttcccac gtcggtcacc tgtcggcgcg
9241 gtgctggtgg gcgagcgcct ccagcacggg tacgacctcg gcgggggtcg gcgcggccag
9301 cgcctcctgc cgcagcttct cggcgttctc ggcgtgggaa cggtcctcga ccactgtggc
9361 gagagcctgc cagagggtgt cggcgtcgac ctcgtccgga cggaggaaga cacccgctcc
9421 cagctcggcg gtgcgctgac cacgcaggac acagtcccac tcgtgggcga cggagatctg
9481 cggtacgccg tggtgcagcg cggtggccca gcttccggca ccgccgtggt ggatgacggc
9541 ggcacagccc ggcagcagga tgttcatggg aacgaagtcc accaggcgga cgttgtccgg
9601 caccgacgcc ggatcgagcc cggagcgggt caccacgatc tcgccgtcga accgcgcgag
9661 ggtggccagt gtccggagga actcctgcgg gttcgaggtg atgcccagcg ccgagtatcc
```

FIG. 7-30

9721	cccggtgaag	cagacccggc	ggactccgtc	cgaggtcctg	agccactgcg	gcacgacgga
9781	ggacccgttg	tagggcaaag	tccgggtgtg	caccgactcc	agtccggtct	ccaggcggaa
9841	gctctcgggc	agctggtcga	cgctccactg	tccgacagcg	aggtcctcgc	tgtagtcgag
9901	gccgaaccgg	ccggcgacct	cggtgagcca	gccgccgagc	gggtccggcc	ggtcgtcggc
9961	gggacgctgc	ccgcgcaggt	cctgggagcg	gctgcggaag	tagccggtga	ggtcgctgcc
10021	ccacagcagc	cgggcgtggg	cggccccgca	ggccttggcc	gcgaccgccc	cggcgaaggt
10081	gaagggctcc	cagagcacca	ggtcgggacg	ccagtccatg	gcgaactcga	cgagttcgtc
10141	gacgaaggag	tcgttgttga	ccaccgggaa	gacgaaccgg	gaggtggcct	cctcgatgcc
10201	gtgcaggaac	tcccacgagc	gcagttccgg	tccgcgtcgg	gcgaagtcca	ggtcggtggt
10261	gtagcggtgc	acctgcgcgg	cggcctcagg	ggagatgtcg	aagagtcggt	ggtccgagcc
10321	gagtggcacc	gaggtcagtc	ccgcgccgac	gacgacgtcg	gtgagctcgg	gctgactggc
10381	cacccggacg	tcgtggccgg	cggtgtgcag	cgcccaggcc	agggggacga	ggccctggaa
10441	gtgggtacgg	tgcgcgaacg	aggtgagcag	gacccgcact	ggtcactcct	tggtcgagat
10501	gagggcggca	acggtccggt	cgatgccctc	ggccagcggc	acccgggggt	gccagccggt
10561	cagcgtccgg	aactcggtgg	agtcgaagtc	gtcgctgcgg	aagtcgttgg	cctcggcgtt
10621	ctccggtgga	gggacgctga	cgacgggcac	cgcagggttg	ccggtctgac	gtgccacgct
10681	ggcggcgacg	gtctcgaaga	tctcgccgag	gggtcgggcc	tcgtccgcgc	tcggcgtcca
10741	gacgtcgccg	accagcgcct	cgtggttgtg	cagtgcggcg	gtgaacgcgg	tggccacgtc
10801	ctcgacgtgc	aggaggttgc	ggcgcacgct	gccctcgtgc	cacatcgtga	tcggctcacc
10861	ggcgagggct	cgccggatca	tggcggtgac	gacaccccgg	ccggtctgcc	ccgacgggcc
10921	gctgtggccg	tagatcgcgg	gcaggcgcag	gatcaccccg	tcgacgaccc	cgtcctcggt
10981	ggcctgacgc	aggatccgct	cggcctcgat	cttgtgctgg	gcgtaccggc	tgggggcggc
11041	ggggttcgcg	gcctgggtgg	tgctggcgaa	caggagcacc	ggcgcgggtc	cgggtcttgc

FIG. 7-31

```
11101 ccgcagcgcg gcgacgaggt cgcgcatgat gcccgcgttg acgcgttcgg cctcgggcac
11161 cgtggcggcg ctgcgccagg tcgacccgcc ggcggcgtag gcgaccagat gcacgacgac
11221 gtcggtgtcg gcgacgacct gcgcgacccg gccgggttcg agcaggtcga ctcgaaggtg
11281 ctcgatcccg gcgctgcctg gtggctggtc gcgagacccg gtgcgcgcga cggcccgcag
11341 tcggagaggg tgtgtggtaa attcgcgaag aagggcgctt ccgacgaatc cagaaacgcc
11401 gagaagtgtg acatgtcttg tcatctacta atgcattccg atagccaccg gcgcatggaa
11461 tccatttgtt ccccccaggg tggtgtcggg tgacaaatcc ggcctcaggt cggcctcaag
11521 cctctttcga gcgggtgctg aggcttcccg cgtaccctcg gtggcctgcg ttcgggcggg
11581 tgtcggggaa agggcggatc gaggagttcg gtagggcgtc gcggcgcgta ctccgggact
11641 gatccgggtc gacgccccga cgcgtgacag ggcgtcgatc cgtgccgccc gtaccgccgg
11701 ttttcggcga tggtcgcaga ttcctcccga cgtggtggac tcattggttc tcccgggtgt
11761 ggccgcaccg tcggtggcct cgtcgggggt gtcggagacc gggtcgatcg ccgtccccgg
11821 ccgtgccgac cagggtcggt ccgtcgccga ggtgggtcac cgtcgggtgg acccggtccg
11881 ccggcggcca ccgcccgatc gtgcccacct tcgcctccgc gggtaaatgc ttcgtcgatc
11941 tgatcgacac ttccggcgac gctatcaccg gagcattccc cggcaccacc ggtcgatgcc
12001 tcgcgctttc caaacaggga aaacagcagc tcacagcggt tccaggcgcc gggcaatcct
12061 agcgaagagt ctcgatgggg tcaaggtgaa ttctgtcaca gatgtttttg ttaaatgtac
12121 tttcttcagc caccctcgac gttcatacaa ttggccggca tctctaccaa gggggagtga
12181 gtggttgacg tgcccgatct actcggcacc cggactccgc acccagggcc gctcccattc
12241 ccgtggcccc tgtgcggtca caacgaaccg gagctgcggg cccgcgcccg tcaattgcac
12301 gcatatctcg aaggcatttc cgaggatgac gtggtggccg tcggcgccgc cctcgcgcgc
12361 gagacacgcg cgcaggacgg gccgcaccgc gccgtcgtcg tggcctcctc ggtcaccgag
12421 ctgaccgccg cgctcgccgc cctcgcccag ggccgcccac acccctcggt ggtacgcggt
```

FIG. 7-32

```
12481 gtcgcccgac ccacggcacc ggtggtgttc gtcctgcccg gtcagggcgc ccagtggccc
12541 ggcatggcga cccgactgct cgccgagtcg cccgtcttcg ccgcggcgat gcgggcctgc
12601 gagcgggcct tcgacgaggt caccgactgg tcgttgaccg aggtcctgga ctcacccgag
12661 cacctgcgcc gcgtcgaggt ggtccagccc gcgctcttcg cggtgcagac ctcactggcc
12721 gccctgtggc ggtcgttcgg ggtgcgaccc gacgccgtac tcggacacag catcggtgag
12781 ctggccgccg ccgaggtctg cggcgccgtc gacgtcgagg ccgccgcgcg ggccgccgcc
12841 ctgtggagcc gcgagatggt cccactggtg ggccggggtg acatggcggc ggtggcgctc
12901 tccccggccg agctggcagc ccgggtcgag cggtgggacg acgacgtcgt gccggccggg
12961 gtcaacggtc cccggtcggt gctgctcacc ggcgctcccg agcccatcgc acggcgggtc
13021 gccgagctgg cggcacaggg cgtacgcgcc caggtcgtca acgtgtcgat ggcggcgcac
13081 tcggcgcagg tcgacgccgt cgccgagggc atgcgctcgg cgctgacctg gttcgccccc
13141 ggcgactccg acgtgcccta ctacgccggc ctcaccggcg ggcggctgga cacccgggaa
13201 ctcggcgccg accactggcc gcgcagtttc cggctcccgg tgcgcttcga cgaggcgacc
13261 cgtgcggtcc tggaactgca gcccggcacg ttcatcgagt cgagcccgca cccggtgctg
13321 gcggcctccc tgcagcagac cctcgacgag gtcgggtccc cggccgcgat cgtgccgacc
13381 ctgcaacgcg accagggcgg tctgcggcgg ttcctgctcg ccgtggcgca ggcgtacacc
13441 ggtggcgtga cagtcgactg gaccgccgcc taccccgggg tgaccccggc ccacctgccg
13501 tcggccgtcg ccgtcgagac cgacgaggga ccctcgacgg agttcgactg ggccgcgccc
13561 gaccacgtac tgcgcgcgcg gctgctggag atcgtcggcg ccgagacggc cgcgctcgcc
13621 gggcgggagg tcgacgcccg ggccaccttc cgggaactgg gcctcgactc ggtcctcgcg
13681 gtgcagctgc ggacccgcct cgccacggcg accgggcggg atctgcacat cgccatgctc
13741 tacgaccacc cgaccccgca cgccctcacc gaggcgctgc tgcgcggccc gcaggaggag
13801 ccgggcgggg gtgaggagac ggcacacccg acggaggccg aacccgacga acccgtcgcc
```

FIG. 7-33

```
13861 gtggtcgcca tggcgtgccg gctgcccggc ggcgtcacct caccggagga gttctgggag
13921 ctgctggccg aggggcggga cgccgtcggc gggctgccca ccgaccgggg atgggacctg
13981 gactcgctgt tccacccgga cccgacccgg tcgggcacgg cgcaccagcg cgctggtggc
14041 ttcctcaccg gcgccacctc cttcgacgct gccttcttcg ggctgtcgcc acgggaggca
14101 ctggccgtcg agccgcagca gcggatcacg ttggagctgt cgtgggaggt gctggaacgc
14161 gccgggatcc ccccgacgtc gttgcggacc tcccggaccg gggtgttcgt cggtctgatc
14221 ccccaggagt acggcccccg gctggccgag gggggtgagg gcgtcgaggg ctacctgatg
14281 accgggacca ccaccagcgt cgcctccggt cgggtcgcct acaccctcgg cctggagggg
14341 ccggcgatca gcgtcgacac cgcctgctcg tcgtcgctcg tcgccgtgca cctggcgtgc
14401 cagtcgctgc ggcgcggcga gtcgacgatg gcgctcgccg gtggcgtgac ggtgatgccg
14461 acaccgggca tgctcgtgga cttcagtcgg atgaactccc tcgcccccga cggacggtcc
14521 aaggcgttct cggccgccgc cgacgggttc ggcatggccg aaggcgcagg gatgctcctg
14581 ctggaacggc tctcggacgc ccgccgccac ggccacccgg tgctcgccgt gatcaggggc
14641 accgctgtca actccgacgg cgcgagcaac ggactctccg ccccgaacgg ccgggcccag
14701 gtccgggtga tccgacaggc cctcgccgag tccgggctga cgccccacac cgtcgacgtc
14761 gtggagaccc acggcaccgg cacccgcctc ggtgatccga tcgaggcacg ggcgctctcc
14821 gacgcgtacg gcggtgaccg tgagcacccg ctgcggatcg gctcggtcaa gtccaacatc
14881 gggcacaccc aggccgccgc cggtgtcgcc ggtctgatca aactggtgtt ggcgatgcag
14941 gccggtgtcc tgccccgcac cctgcacgcc gacgagccgt caccggagat cgactggtcc
15001 tcgggcgcga tcagcctgct ccaggagccc gctgcctggc ccgccggcga gcggccccgc
15061 cgggccgggg tgtcctcgtt cggcatcagc ggcaccaacg cacacgcgat catcgaggag
15121 gcgccgccga ccggtgacga cacccgaccc gaccggatgg gcccggtggt gccctgggtg
15181 ctctcggcga gcaccggcga ggcgttgcgc gcccgggcgg cgcggctggc cgggcaccta
```

FIG. 7-34

15241 cgcgagcacc ccgaccagga cctggacgac gtcgcctact cgctggccac cggtcgggcc
15301 gcgctggcgt accgtagtgg gttcgtgccc gccgacgcgt ccacggcgct gcggatcctc
15361 gacgaactcg ccgccggtgg atccggggac gcggtgaccg gcaccgcccg cgccccgcag
15421 cgcgtcgtct tcgtcttccc cggccaggga tggcagtggg cggggatggc agtcgacctg
15481 ctcgacggcg acccggtctt cgcctcggtg ctgcgggagt gcgccgacgc gttggaaccg
15541 tacctggact tcgagatcgt cccgttcctg cgggccgagg cgcagcgccg gacccccgac
15601 cacacgctct ccaccgaccg cgtcgacgtg gtccagccgg tgctgttcgc ggtgatggtg
15661 tccctggcgg cccggtggcg ggcgtacggg gtggaaccgg cggccgtcat cggacactcc
15721 cagggggaga ttgccgcggc gtgtgtggcc ggggcgctct cgctggacga cgcggcccgg
15781 gcggtggccc tgcgcagccg ggtcatcgcc accatgcccg gcaacggcgc gatggcctcg
15841 atcgccgcct ccgtcgacga ggtggcggcc cggatcgacg ggcgggtcga gatcgccgcc
15901 gtcaacggtc cgcgcgcggt ggtggtctcc ggcgaccgtg acgacctgga ccgcctggtc
15961 gcctcctgca ccgtcgaggg ggtgcgggcc aagcggctgc cggtggacta cgcgtcgcac
16021 tcctcgcacg tcgaggccgt ccgtgacgcg ctccacgccg aactcggcga gttccggccg
16081 ctgccgggct tcgtgccgtt ctactcgaca gtcaccggcc gctgggtcga gcccgccgaa
16141 ctcgacgccg ggtactggtt tcgcaacctg cgccacaggg tccggttcgc cgacgcggtc
16201 cgctccctcg ccgaccaggg gtacacgacg ttcctggagg tcagcgccca cccggtgctc
16261 accacggcga tcgaggagat cggtgaggac cgtggcggtg acctcgtcgc tgtccactcg
16321 ctgcgacgtg gggccggcgg tcccgtcgac ttcggctccg cgctggcccg cgccttcgtg
16381 gccggcgtcg cagtggactg ggagtcggcg taccagggtg ccggggcgcg tcgggtgccg
16441 ctgcccacgt acccgttcca gcgtgagcgc ttctggttgg aaccgaatcc ggcccgcagg
16501 gtcgccgact ccgacgacgt ctcgtccctg cggtaccgca tcgaatggca cccgaccgat
16561 ccgggtgagc cgggacggct cgacggcacc tggctgctgg cgacgtaccc cggtcgggcc

FIG. 7-35

```
16621 gacgaccggg tcgaggcggc gcggcaggcg ctggagtccg ccggggcgcg ggtcgaggac
16681 ctggtggtgg agccccggac gggccgggtc gacctggtgc ggcggctcga cgccgtgggt
16741 ccggtggcgg gcgtgctctg cctgttcgct gtcgcggagc cggcggccga acactccccg
16801 ctggcggtga cgtcgttgtc ggacacgctc gacctgaccc aggcggtggc cgggtcgggc
16861 cgggagtgtc cgatctgggt ggtcaccgag aacgccgtcg ccgtcgggcc cttcgaacgg
16921 ctccgcgacc cggcccacgg cgcgctctgg gccctcggtc gggtcgtcgc cctggagaac
16981 cccgccgtct ggggcggcct ggtcgacgtg ccgtcgggtt cggtcgccga gctgtcgcgt
17041 cacctcggga cgaccctgtc cggcgccggc gaggaccagg tcgccctccg acccgacggg
17101 acgtacgccc gccggtggtg cagggcgggc gcgggcggca cgggccggtg gcagccccgg
17161 ggcacggtgc tcgtcaccgg cggcaccggc ggggtcggtc ggcacgtcgc ccggtggctg
17221 gcccgccagg gcaccccgtg cctggtgctg gccagccgcc ggggaccgga cgccgacggg
17281 gtcgaggagc tactcaccga actcgccgac ctgggcaccc gggccaccgt caccgcctgc
17341 gacgtcaccg accgggagca gctccgtgcc ctcctcgcga ccgtcgacga cgagcacccg
17401 ctgtcggcgg tgttccacgt cgccgcgacg ctcgacgacg gcaccgtcga gaccctcacc
17461 ggtgaccgca tcgaacgggc caaccggcg aaggtgctcg gtgcccgcaa cctgcacgag
17521 ctgacccggg acgccgacct cgacgcgttc gtgctcttct cctcctccac cgccgcgttc
17581 ggcgcgccgg ggctcggcgg ctacgtcccg ggcaacgcct acctcgacgg tctcgcccag
17641 cagcgacgca gcgagggact cccggccacc tcggtggcgt ggggtacctg ggcgggcagc
17701 gggatggccg agggtccggt cgccgaccgg ttccgccggt acggggtcat ggagatgcac
17761 cccgaccagg ccgtcgaggg tctccgggtg gcactggtgc agggtgaggt agccccgatc
17821 gtcgtcgaca tcaggtggga ccggttcctc ctcgcgtaca ccgcgcagcg ccccacccgg
17881 ctcttcgaca ccctcgacga ggcccgtcgg gccgcgcccg gtcccgacgc cgggccgggg
17941 gtggcggcgc tggccgggct gcccgtcggg gaacgcgaga aggcggtcct cgacctggta
```

FIG. 7-36

```
18001 cggacgcacg cggctgccgt cctcggccac gcctcggccg agcaggtgcc cgtcgacagg
18061 gccttcgccg aactcggcgt cgactcgctg tcggccctgg aactgcgcaa ccggctgacc
18121 actgcgaccg gggtccggct ggccacgacg acggtcttcg accacccgga cgtacggacc
18181 ctggccggac acctggccgc cgaactgggc ggcggatcgg ggcgggagcg gcccgggggc
18241 gaggccccga cggtggcccc gaccgacgag ccgatcgcca tcgtcgggat ggcctgccgg
18301 ctgccggggg gagtggactc accggagcag ctgtgggagt tgatcgtctc cgggcgggac
18361 accgcctcgg cggcacccgg ggaccggagc tgggatccgg cggagttgat ggtctccgac
18421 acgacgggca cccgtaccgc cttcggcaac ttcatgcccg gggcggcgga gttcgacgcg
18481 gcgttcttcg ggatctcgcc gcgtgaggcg ttggcgatgg atccgcagca gcggcacgcc
18541 ctggagacca cctgggaggc gctggagaac gccggtatcc ggcccgagtc gttgcgcggt
18601 acggacaccg gtgtcttcgt gggcatgtcc catcaggggt acgccaccgg ccgcccgaag
18661 cccgaggacg aggtcgacgg ctacctgttg acaggcaaca ccgcgagcgt cgcctccggt
18721 cggatcgcgt acgtgttggg gttggagggg ccggcgatca ctgtggacac ggcgtgttcg
18781 tcgtcgcttg tggcgttgca cgtggcggcg ggttcgttgc gttctgggga ctgtggtctg
18841 gcggtggcgg gtggggtgtc ggtgatggcc ggtccggagg tgttcaggga gttctcccgg
18901 cagggcgcgt tggctccgga cggcaggtgc aagcccttct cggacgaggc cgacggcttc
18961 ggtctggggg aggggtcggc cttcgtcgtg ttgcagcggt tgtcggtggc ggtgcgggag
19021 gggcgtcggg tgttgggtgt ggtggtgggt tcggcggtga atcaggatgg ggcgagtaat
19081 gggttggcgg cgccgtcggg ggtggcgcag cagcgggtga ttcggcgggc gtggggtcgt
19141 gcgggtgtgt cgggtgggga tgtgggtgtg gtggaggcgc atgggacggg gacgcggttg
19201 ggggatccgg tggagttggg ggcgttgttg gggacgtatg gggtgggtcg gggtggggtg
19261 ggtccggtgg tggtgggttc ggtgaaggcg aatgtgggtc atgtgcaggc ggcggcgggt
19321 gtggtgggtg tgatcaaggt ggtgttgggg ttgggtcggg ggttggtggg tccgatggtg
```

FIG. 7-37

```
19381 tgtcggggtg ggttgtcggg gttggtggat tggtcgtcgg gtgggttggt ggtggcggat
19441 ggggtgcggg ggtggccggt gggtgtggat ggggtgcgtc ggggtggggt gtcggcgttt
19501 ggggtgtcgg ggacgaatgc tcatgtggtg gtggcggagg cgccggggtc ggtggtgggg
19561 gcggaacggc cggtggaggg gtcgtcgcgg gggttggtgg gggtggttgg tggtgtggtg
19621 ccggtggtgc tgtcggcaaa gaccgaaacc gccctgcacg cccaggcacg tcgactcgcc
19681 gaccacctgg agacgcaccc cgacgtcccg atgaccgacg tggtgtggac gctgacgcag
19741 gcccgccaac gcttcgacag gcgcgcggtc ctcctcgccg ccgaccggac ccaggccgtg
19801 gaacggctgc gcggcctcgc cgggggcgaa ccggggaccg gtgtggtgtc gggggtggcg
19861 tcgggtggtg gtgtggtgtt tgtttttcct ggtcagggtg gtcagtgggt ggggatggcg
19921 cgggggttgt tgtcggttcc ggtgtttgtg gagtcggtgg tggagtgtga tgcggtggtg
19981 tcgtcggtgg tggggttttc ggtgttgggg gtgttggagg gtcggtcggg tgcgccgtcg
20041 ttggatcggg tggatgtggt gcagccggtg ttgttcgtgg tgatggtgtc gttggcgcgg
20101 ttgtggcggt ggtgtggggt tgtgcctgcg gcggtggtgg gtcattcgca gggggagatc
20161 gcggcggcgg tggtggcggg ggtgttgtcg gtgggtgatg gtgcgcgggt ggtggcgttg
20221 cgggcgcggg cgttgcgggc gttggccggc cacggcggca tggcctcggt acgccgaggc
20281 cgcgacgacg tacagaagct cctcgacagc ggcccctgga cggggaagct ggagatcgcc
20341 gcggtcaacg gccccgacgc ggtggtggtc tccggcgacc cccgagccgt gaccgagctg
20401 gtcgagcact gtgacgggat cggggtccgg gcccggacga tccccgtcga ctacgcctcc
20461 cactccgcac aggtcgagtc gctccgggag gagctgctct ccgtcctggc cgggatcgag
20521 ggccgcccgg cgacggtgcc gttctactcc accctcaccg gtgggttcgt cgacggcacc
20581 gaactggacg ccgactactg gtaccgcaac ctgcgccacc cggtgcggtt ccacgccgcc
20641 gtcgaggcgc tggcagcgcg tgacctcacc acgttcgtcg aggtcagccc gcaccccgtg
20701 ctgtcgatgg cggtcgggga gacgcttgcc gacgtggagt ccgccgtcac tgtgggcacc
```

FIG. 7-38

```
20761 ctggaacgcg acaccgacga cgtcgagcgc ttcctcacct ccctcgccga ggcgcacgtc
20821 cacggcgtac ccgtggactg ggcggcggtc ctcggctccg gaaccctggt cgacctgccc
20881 acctatccct tccagggacg gcggttctgg ctgcaccccg accgtggtcc gcgtgacgat
20941 gtcgccgact ggttccaccg ggtcgactgg acggcgacgg ccaccgacgg gtcggcccga
21001 ctcgacggtc gctggctggt ggtcgtaccc gaggggtaca cggacgacgg ctgggtcgtg
21061 gaggtgcggg ccgccctcgc cgccggtggt gccgagccgg tggtgacgac ggtcgaggag
21121 gtcaccgacc gggtcggtga cagcgacgcg gtggtgtcga tgctcgggct ggccgacgac
21181 ggtgcggccg agaccctggc gctgctgcga cgactcgacg cacaggcgtc caccacccca
21241 ctgtgggtgg tcaccgtggg ggccgtcgcc cccgccggtc cggtgcagcg ccccgaacag
21301 gcgacggtgt gggggttggc ccttgtcgcc tccctggaac gcggacaccg gtggaccggc
21361 ctgctggatc tgccgcagac accggacccg cagctacgac cccggctggt cgaggcgctc
21421 gccggtgccg aggaccaggt agcggtccgc gccgacgccg tacacgcccg tcggatcgtc
21481 cccaccccgg tcaccggagc cgggccgtac accgccccgg gcgggacgat cctcgtcacc
21541 gggggcaccg ccggtctggg tgccgtcacc gcccgatggc tcgccgagcg cggtgccgaa
21601 cacctcgccc tggtcagccg gcgcgggccg ggcaccgccg gcgtcgacga ggtggtccgg
21661 gacctgaccg ggctcggcgt acgggtgtcg gtgcactcct gcgacgtcgg cgaccgcgag
21721 tcggtcggcg ccctggtgca ggagttgaca gcagccggtg acgtggtccg gggggtggtc
21781 cacgctgccg gtctgcccca gcaggtgcca ctgaccgaca tggacccggc cgacctcgcc
21841 gacgtggtgg ccgtgaaggt cgacggcgcg gtgcacctgg ccgacctgtg cccggaggcc
21901 gaactgttcc tgctgttctc ctccggggcc ggggtgtggg gcagtgcccg tcagggtgcg
21961 tacgccgccg gaaacgcctt cctggacgcc ttcgcccgac accggcggga ccggggtctg
22021 cccgccacct cggtggcgtg gggctctgg gcggccgggg ggatgacagg ggaccaggag
22081 gcggtgtcgt tcctgcgtga gcggggcgta cggccgatgt cggtgccgag ggcactggaa
```

FIG. 7-39

```
22141 gcgctggaac gggtcctcac cgccggggag accgcggtgg tcgtcgccga cgtcgactgg
22201 gcggccttcg ccgagtcgta cacctccgcc cggccccggc cgctgctcca ccggctcgtc
22261 acacctgcgg cggcggtcgg cgagcgcgac gagccgcgtg agcagaccct ccgggaccgg
22321 ctggcggccc tgccccgggc cgagcggtcg gcggagctgg tacgcctggt ccggcgggac
22381 gccgcagccg tgctcggcag cgacgcgaag gccgtacccg ccaccacgcc gttcaaggac
22441 ctcgggttcg actcgctggc cgcggtccgg ttccgtaacc ggctggccgc ccacaccggt
22501 ctgcgtctgc cggccaccct ggtcttcgag cacccgaacg ccgcagccgt cgccgacctc
22561 ctccacgacc gactcggcga ggccggcgag ccgacccccg tccggtcggt gggcgccgga
22621 ctggccgcgc tggagcaggc cctgcccgac gcctccgaca cggagcgggt cgagctggtc
22681 gagcgcctgg aacggatgct cgccgggctc cgccccgagg ccggagccgg ggccgacgcc
22741 ccgaccgccg gtgacgacct gggggaggcc ggcgtcgacg aactcctcga cgcgctcgaa
22801 cgggaactcg acgccaggtg aacccgaact gaccgcagcc gcagccgaag cagagaccga
22861 ggacctgtga ctgacaacga caaggtggcg gagtacctcc gtcgtgcgac gctcgacctg
22921 cgggccgccc gcaagcgcct gcgcgagctg caatccgacc cgatcgcggt cgtcggcatg
22981 gcctgccgcc taccgggcgg ggtgcacctc ccgcagcacc tgtgggacct cctgcgccag
23041 gggcacgaga cggtgtccac cttccccacc gggcgcggct gggacctggc cgggctcttc
23101 cacccggacc ccgaccaccc cggcaccagc tacgtcgacc ggggtgggtt cctcgacgac
23161 gtggcgggct tcgacgccga gttcttcggg atctccccgc gcgaggccac ggccatggac
23221 ccgcaacagc ggctgctgtt ggagaccagt tgggagctgg tggagagcgc cggcatcgat
23281 ccgcactccc tgcgtggcac cccgaccggc gtcttcctcg gcgtggcgcg gctcggctac
23341 ggcgagaacg gcaccgaagc cggtgacgcc gagggctatt cggtgaccgg ggtggcaccc
23401 gctgtcgcct ccgggcggat ctcctacgcc ctcgggctgg agggtccgtc gatcagcgtg
23461 gacaccgcgt gctcgtcgtc gttggtggcg ctgcacctgg cggtcgagtc gctgcggctg
```

FIG. 7-40

23521 ggcgagtcga gtctcgctgt cgtcggcggg gcggcggtca tggcgacacc aggggtgttc
23581 gtcgacttca gccgccagcg ggcgttggcc gctgacggca ggtcgaaggc cttcggggcc
23641 gccgccgacg ggttcggctt ctccgagggg gtctccctcg tcctgctcga acggctctcc
23701 gaggccgaaa gcaacggcca cgaggtgttg gctgtcatcc gtggctccgc cctcaaccag
23761 gacggggcca gcaacggtct cgccgcgccg aacgggaccg cccagcgcaa ggtgatccgg
23821 caggcgctac gaaactgcgg cctgaccccg gccgacgtgg acgccgtgga ggcgcacggc
23881 accggcacca cgctcggcga cccgatcgag gccaacgccc tgctggacac ctacggccgt
23941 gaccgggatc cggaccaccc gctgtggctg gggtcggtga agtcgaacat cggccacacg
24001 caggcggcgg cgggcgtcac cgggctgctc aagatggtgc tggcactgcg ccacgaggaa
24061 ctgcccgcca ccctgcacgt cgacgagccc accccgcacg tggactggtc ctcgggagcg
24121 gtacgcctgg cgacccgggg ccggccgtgg cggcggggtg accggccgag gcgggccggg
24181 gtgtcggcgt tcggcatcag cgggaccaac gcccacgtga tcgtcgagga ggcacccgag
24241 cggaccaccg agcgcaccgt cggcggcgac gtcggcccgg tcccgctcgt ggtgtccgcc
24301 cggtcggcgg cggcgctacg ggcccaggcg gcccaggtcg ccgagctggt ggagggctcc
24361 gacgtcgggc tggcggaggt cgggcggagc ctggccgtga cccgggcgcg acacgagcac
24421 cgggcggcgg tggtggcgtc gacccgggcc gaggcggtgc gggggctgcg cgaggtcgcg
24481 gcggtcgaac cgcgcggcga ggacaccgtc accggggtcg ccgagacgtc cgggcgcacc
24541 gtcgtcttcc tcttcccggg acaggggtcc cagtgggtcg ggatgggcgc ggagctgctg
24601 gactcggcac cggcgttcgc cgacacgatc cgcgcctgcg acgaggcgat ggcaccgttg
24661 caggactggt cggtctccga cgtgctccgg caggagccgg gggcaccggg actggaccgg
24721 gtcgacgtgg tgcagccggt gctgttcgcg gtgatggtgt cgttggcgcg gttgtggcag
24781 tcgtacgggg tcacccccgc tgcggtggtg gggcactcgc agggggagat cgccgccgcc
24841 cacgtggcgg gtgcgctctc cctcgccgac gcggcgaggc tggtggtggg ccgcagccgg

FIG. 7-41

```
24901 ttgctgcggt cgctgtccgg gggcggcggc atgagcgccg tcgcgctcgg tgaggccgag
24961 gtacgccgcc gactgcggtc gtgggaggac cggatctccg tggccgccgt caacggaccc
25021 cggtcggtgg tggtggccgg ggaaccggag gcgctgcggg agtggggacg ggagcgggag
25081 gccgagggcg tacgggtccg cgagatcgac gtcgactacg cctcgcactc gccgcagatc
25141 gacagggtcc gtgacgaact cctgacggtc acgggggaga tcgagccccg gtcggcggag
25201 atcaccttct actcgacggt cgacgtccgt gctgtcgacg gcaccgacct ggacgcgggg
25261 tactggtacc gcaacctgcg ggagacggtc cggttcgccg acgcgatgac ccggttggcc
25321 gactcgggat acgacgcgtt cgtcgaggtc agcccgcatc cggtggtggt gtcggcggtc
25381 gccgaggcgg tcgaggaggc aggtgtcgag gacgccgtcg tcgtcggcac cctgtcccgg
25441 ggcgacggcg gaccgggggc gttcctgcgg tcggcggcca ccgcccactg cgccggtgtg
25501 gacgtcgact ggacgcccgc cctcccggga gctgcgacga tcccgttgcc gacgtacccg
25561 ttccaacgga agccgtactg gctgcggtcg tctgctcccg cccccgcctc ccacgatctc
25621 gcctaccggg tgtcctggac gccgatcacc ccgcccgggg acggcgtact cgacggcgac
25681 tggctggtgg tgcaccccgg gggcagcacc ggatgggtcg acgggttggc ggcggcgatc
25741 accgccggcg gtggccgggt cgtcgcccac ccggtggact ccgtgacctc ccggaccggc
25801 ctggccgagg cgctcgcccg gcgggacggc acgttccggg gggtgctgtc gtgggtggcg
25861 accgacgaac ggcacgtcga ggccggtgcg gtcgccctgc tgaccctggc gcaggcgttg
25921 ggtgacgccg gaatcgacgc accactgtgg tgcctgaccc aggaggcggt ccgtacccc
25981 gtcgacggtg acctggcccg accggcgcag gccgccctgc acggtttcgc ccaggtcgcc
26041 cggctggagc tggcccgccg cttcggtggg gtgctcgacc tgcccgccac cgtcgacgcc
26101 gccgggacgc gtctggtcgc ggcggtcctc gccggcggcg gcgaggacgt cgtcgccgtc
26161 cgtggcgacc gtctctacgg ccgtcgcctg gtcagggcga ccctgccgcc gcccggcggg
26221 gggttcaccc cgcacggcac cgtcctggtc accggcgcgg ccggtccggt gggcggtcgg
```

FIG. 7-42

```
26281 ctggcccggt ggctcgccga acggggtgcc acccgactcg tcctgcccgg cgcacacccg
26341 ggcgaggagt tgctgaccgc gatccgggcc gccggtgcca ccgccgtggt gtgcgaaccg
26401 gaggcggagg cactgcgtac ggcgatcggc ggggagttgc cgaccgcgct cgtacacgcc
26461 gagacgttga cgaacttcgc cggcgtcgcc gacgccgacc ccgaggactt cgccgccacc
26521 gtcgcggcga agaccgcgct gccgacggtc ctggcggagg tgctcggcga ccaccgcctc
26581 gaacgggagg tctactgctc gtcggtggcc ggggtctggg gtggggtcgg catggccgcg
26641 tacgccgccg gcagcgccta cctcgacgcc ctggtcgagc accgtcgcgc ccgggggcac
26701 gccagcgcct cggtggcctg gaccccgtgg gccctgcccg gcgcggtcga cgacggtcgg
26761 ctgcgcgagc gcggcctgcg cagcctcgac gtggccgacg ccctcgggac gtgggaacgt
26821 ctgctccgcg ccggtgcggt gtcggtggcc gtcgccgacg tcgactggtc ggtcttcaca
26881 gagggtttcg cggccatccg gccgaccccg ctcttcgacg aactcctcga ccggcgcggg
26941 gaccccgacg gcgcgcccgt cgaccggccg ggggagccgg cgggcgagtg gggtcgacga
27001 atcgcggcgc tgtccccgca ggaacagcgg gagacgttgc tgaccctcgt cggcgagacg
27061 gtcgcggagg tgctgggaca cgagaccggc accgagatca acacccgtcg ggccttcagc
27121 gaactcggcc tcgactcgct gggctcgatg gccctgcgtc agcgcctggc ggcccgtacc
27181 ggcctgcgga tgccggcctc gctggtctta gaccacccga cggtcaccgc gctcgcgcgg
27241 tacctgcgtc gactggtcgt cggggactcc gacccgaccc cggtacgggt gttcggcccc
27301 accgacgagg ccgaacccgt cgccgtggtc ggcatcggct gccggttccc cggcggcatc
27361 gccacccccg aggacctctg gcgggtggtg tccgagggca cctccatcac caccggattc
27421 cccaccgacc ggggctggga cctccggcgg ctctaccacc ccgacccgga ccaccccggc
27481 accagctacg tcgacagggg gggattcctc gacggggccc cggacttcga ccccgggttc
27541 ttcgggatca ccccccgcga ggcgctggcg atggacccgc agcagcggct caccctggag
27601 atcgcgtggg aggcggtgga acgggcgggc atcgacccgg agaccctcct cggcagcgac
```

FIG. 7-43

27661	accggcgtct	tcgtcggcat	gaacggccag	tcctacctgc	aactgctgac	cggggagggt
27721	gaccggctca	acggctacca	ggggttgggc	aactcggcga	gcgtgctctc	cggccgtgtc
27781	gcctacacct	tcgggtggga	ggggccggcg	ctgacggtgg	acaccgcctg	ctcgtcctcg
27841	ctggtcgcca	tccacctcgc	catgcagtcg	ctgcgtcggg	gtgagtgctc	gctggcgttg
27901	gccggcgggg	tgacggtcat	ggccgacccg	tacaccttcg	tggacttcag	cgcacagcgg
27961	gggctcgccg	ccgacgggcg	gtgcaaggcg	ttctccgcgc	aggccgacgg	gttcgccctc
28021	gccgagggcg	tcgcggcgct	cgtcctcgaa	ccgttgtcca	aggcgcggcg	aaacggccac
28081	caggtgctgg	cggtgctgcg	cggcagcgcc	gtcaaccagg	acggggccag	caacggcctc
28141	gccgccccga	acgggccgtc	gcaggaacgg	gtgatcaggc	aggccctgac	cgcctccggg
28201	ctgcgtcccg	ccgacgtcga	catggtggag	gcgcacggga	cgggcaccga	actcggcgac
28261	ccgatcgagg	ccggggcgct	catcgcggcg	tacggccggg	accgggaccg	gccgctctgg
28321	ctgggctcgg	tgaagacgaa	catcggccac	acccaggccg	ccgccggtgc	cgccggggtg
28381	atcaaggcgg	tcctggcgat	gcggcacggc	gtactcccga	ggtcgctgca	cgccgacgag
28441	ttgtccccgc	acatcgactg	ggcggacggg	aaggtcgagg	tgctccgcga	ggcacgacag
28501	tggcccccg	gtgagcgccc	ccgccgcgcc	ggggtgtcct	ccttcggcgt	cagcgggacc
28561	aacgcccacg	tcatcgtcga	ggaggcaccc	gccgaaccgg	accccgaacc	ggttcccgcc
28621	gccccgggcg	ggcccctgcc	cttcgtcctg	cacggacgca	gcgtccagac	ggtccggtcc
28681	caggcgcgga	ccctcgccga	acacctgcgc	accaccggcc	accgggacct	cgccgacacc
28741	gcccgtaccc	tggccaccgg	tcgcgcccgt	ttcgacgtcc	gggccgcagt	gctcggcacc
28801	gaccgggagg	gtgtctgcgc	cgccctcgac	gcgctggcgc	aggatcgccc	ctcgcccgac
28861	gtcgtcgccc	cggcggtctt	cgccgcccgt	acccccgtcc	tggtcttccc	cgggcagggg
28921	tcgcagtggg	tcggcatggc	ccgtgacctg	ctcgactcct	ccgaggtgtt	cgccgagtcg
28981	atgggccggt	gcgccgaggc	gctgtcgccg	tacaccgact	gggacctgct	cgacgtggtc

FIG. 7-44

29041 cgtggggtcg gcgaccccga cccgtacgac cgggtggacg tgctccagcc ggtgctgttc
29101 gcggtgatgg tgtcgctggc gcggttgtgg cagtcgtacg gggtgactcc gggtgcggtg
29161 gtgggtcact cgcaggggga gatcgccgcc gcgcacgtgg ctggtgcgtt gtcgttggcc
29221 gacgccgcca gggtggtggc gttgcgcagc cgggtgctgc gggagctcga cgaccagggc
29281 ggcatggtgt cggtcggcac ctcccgcgcc gagttggact cggtcctgcg ccggtgggac
29341 gggcgggtcg cggtggcggc ggtgaacgga cccggcacgc tcgtggtggc cggacccacc
29401 gccgaactgg acgagttcct cgcggtggcc gaggcccgcg agatgaggcc gcgtcggatc
29461 gcggtgcgct acgcgtcgca ctccccggag gtggcccggg tcgaacagcg gctcgccgcc
29521 gaactcggca ccgtcaccgc cgtcggcggc acggtcccgc tctactccac cgccaccggg
29581 gacctcctcg acaccacagc catggacgcc gggtactggt accgcaacct gcgccaaccg
29641 gtgctgttcg agcacgccgt ccgcagcctc ctggagcggg gattcgagac gttcatcgag
29701 gtcagcccgc accctgtgct gctgatggcg gtcgaggaga ccgccgagga cgccgagcgc
29761 ccggtcaccg gcgtgccgac gctgcgccgc gaccacgacg ggccgtcgga gttcctccgc
29821 aacctcctgg gggcgcacgt gcacggggtc gacgtcgacc tgcgtccggc ggtcgcccac
29881 ggccgcctgg tcgacctgcc cacctacccc ttcgacaggc agcggctctg gcccaagccg
29941 caccgcaggg ccgacacctc gtcgctgggg gtccgtgact cgacccaccc gctgctgcac
30001 gccgcagtcg acgtacccgg tcacggcgga gcggtgttca ccgggcggct ctcccccgac
30061 gagcagcagt ggctgaccca gcacgtggtg ggtgggcgga acctggtgcc cggcagtgtc
30121 ctggtcgacc tcgcgctcac cgccggggcc gacgtcggcg tgccggtgct ggaggaactc
30181 gtcctgcagc agccgctggt gttgaccgcc gccggtgcgt tgctgcgcct gtcggtcggc
30241 gccgccgacg aggacggcg gcggccggtc gagatccacg ccgccgagga cgtctccgac
30301 ccggccgagg cccggtggtc ggcgtacgcg accgggaccc tcgccgtcgg cgtggccggc
30361 ggcggccggg acggcacaca gtggccccg cccggcgcca ccgccctgac gttgaccgac

FIG. 7-45

```
30421 cactacgaca ccctcgccga actgggctac gagtacgggc cggcgttcca ggcgctgcgc
30481 gccgcgtggc agcacggcga cgtggtctac gcggaggtgt ccctcgacgc cgtcgaggag
30541 gggtacgcgt tcgacccggt gctgctcgac gccgtcgccc agaccttcgg cctgaccagt
30601 cgcgcccccg ggaagctccc cttcgcctgg cggggcgtca ccctgcacgc caccggggcc
30661 actgcggtac gggtggtggc gacccccgcc ggaccggacg cggtggccct gcgggtcacc
30721 gacccgaccg gtcagctcgt cgccacggtg gacgccctgg tcgtcaggga cgccggggcg
30781 gatcgggacc agccgcgcgg ccgcgacggc gacctgcacc gcctggagtg ggtacggctg
30841 gccaccccgg acccgacccc ggcggcggtg gtgcacgtgg cggccgacgg gctcgacgac
30901 ctgctgcgcg ccggtggtcc ggcaccacag gccgtcgtcg tccgctaccg tcccgacggc
30961 gacgacccga cggccgaggc ccgtcacggg gtgctctggg cggccacgct cgtgcgccgt
31021 tggctcgacg acgaccggtg gcccgccacc accctggtgg tggccacgtc cgcaggggtc
31081 gaggtctccc ccggggacga cgtgccgcgc cccggggccg ccgccgtgtg gggggtgctg
31141 cgctgcgccc aggcggagtc cccggaccgc ttcgtgctcg tcgacggcga cccggagacg
31201 cccccggcgg tgccggacaa tccgcagctc gcggtccgtg acggtgcggt gttcgtgcca
31261 cggctgacgc cgctcgccgg tcccgtgccg gccgtcgccg accgggcgta ccggctggtg
31321 cccggcaaca gcggctccat cgaggcagtg gccttcgccc ccgtccccga cgccgaccgg
31381 cccctggcgc cggaggaggt acgcgtcgcc gtccgcgcca ccggcgtgaa cttccgtgac
31441 gtcctgctcg cgctcggcat gtacccggaa ccggccgaga tgggcaccga ggcgtccggt
31501 gtggtcaccg aggtcgggtc gggtgtccgg cggttcaccc ccggccaggc ggtgacgggc
31561 ctgttccagg gggccttcgg gccggtggcg gtcgccgacc accggctcct caccccggtc
31621 cccgacgggt ggcgggcggt ggacgccgca gccgtaccca tcgcgttcac caccgcccac
31681 tacgcgctgc acgacctggc cgggttgcag gccgggcagt ccgtgctggt ccacgccgcc
31741 gccggcgggg tggggatggc tgccgtcgcg ttggcccgtc gggccggggc ggaggtgttc
```

FIG. 7-46

```
31801 gccacggcca gcccggccaa acacccgacg ctgcgggcgc tcggcctcga cgacgaccac
31861 atcgcctcgt cccgggagag cgggttcggt gagcggttcg ccgcgcgtac cgggggcgg
31921 ggcgtcgacg tggtcctgaa ctcgctcacc ggcgacctgc tcgacgagtc cgcgcggctg
31981 ctcgccgacg gcggggtctt cgtcgagatg ggcaagaccg acctgcggcc ggcggagcag
32041 ttccgggcc ggtacgtccc gttcgacctg gccgaggccg gtcccgatcg gctcggcgag
32101 atcctggagg aggtcgtcgg tctgctggcc gccggtgccc tcgaccggtt gccggtgtcg
32161 gtgtgggagt tgtcggcggc cccggccgcg ctcacccaca tgagccgggg ccgacacgtg
32221 ggcaagctcg tcctcaccca gcccgccccc gtgcaccccg acggaacggt gctggtcacc
32281 ggcgggaccg gcaccctggg gcggctggtc gcccgccacc tggtgaccgg gcacggcgta
32341 ccccacctcc tggtggccag ccggcgcggt ccggcggccc cgggcgcggc cgagctgcgc
32401 gccgacgtcg aaggcctcgg cgcgaccatc gagatcgtcg cctgcgacac cgccgaccgg
32461 gaggcgctcg cggcgctgct cgactcgatc cccgcggacc gtccgctgac cggggtggtg
32521 cacaccgccg gggtcctggc cgacgggctg gtcacctcca tcgacgggac cgccaccgat
32581 caggtcctgc gggccaaggt cgacgcggcg tggcacctgc acgacctgac ccgggacgcg
32641 gacctgagct tcttcgtgct gttctcgtcg gcggcgtcgg tgctggccgg tcccgggcag
32701 ggcgtgtacg cggcggccaa cggggtcctc aacgccctgg ccgggcaacg gcgggccctc
32761 ggactgcccg cgaaggcgct cgggtggggc ctgtgggcgc aggccagcga gatgaccagc
32821 ggcctcggtg accggatcgc ccgtaccggg gtcgccgcgc tgccgaccga gcgggcgctg
32881 gccctgttcg acgcggctct gcgcagcggc ggggaggtgc tgttcccgct gtctgtcgac
32941 aggtcggcgc tgcgccgggc cgagtacgtc cccgaggtgc tgcgcggcgc ggtccggtcc
33001 acgccacggg ccgccaacag ggccgagacc ccgggccggg gcctgctcga ccgtctcgtc
33061 ggtgcacccg agaccgatca ggtggccgcg ctggccgagc tggtccgctc gcacgcggcg
33121 gcggtcgccg gctacgactc ggccgaccag ctgcccgaac gcaaggcgtt caaggacctc
```

FIG. 7-47

33181	gggttcgact	cgctggcggc	ggtggagctg	cgcaaccggc	tcggcgtcac	caccggcgta
33241	cggctgccca	gcacgctggt	gttcgaccac	ccgacaccgc	tggcggtggc	cgaacacctg
33301	cggtcggagt	tgttcgccga	ctccgcgccg	gacgtcgggg	tcggtgcgcg	cctcgacgac
33361	ctggaacggg	cgctcgacgc	cctgcccgac	gcgcagggac	acgccgacgt	cggggcccgc
33421	ctggaggcgc	tgctgcgccg	gtggcagagc	cgacgacccc	cggagaccga	gccagtgacg
33481	atcagtgacg	acgccagtga	cgacgagctg	ttctcgatgc	tcgacaggcg	tctcggcggg
33541	ggaggggacg	tctaggtgac	aggtcgattc	cgccccgcgg	cagtggaccg	taccgccctg
33601	acaggtccac	cgggttcgcg	tcgcctccca	cacccgacgg	ccggggtatc	cacggaaggg
33661	atccgatgag	cgagagcagc	ggcatgaccg	aggaccgcct	ccggcgctat	ctcaagcgca
33721	ccgtcgccga	actcgactcg	gtgacaggtc	ggctcgacga	ggtcgagtac	cgggcccgcg
33781	aaccgatcgc	cgtcgtcggc	atggcctgcc	ggttccccgg	gggtgtggac	tcgccggagg
33841	cgttctggga	gttcatccgc	gacggtggtg	acgcgatcgc	cgaggcgccc	acggaccgtg
33901	gctggccgcc	ggcaccgcga	ccccgcctcg	gtggtctcct	cgcggagccg	ggcgcgttcg
33961	acgccgcctt	cttcggcatc	tcaccccgcg	aggcgctcgc	gacggacccc	cagcagcgcc
34021	tgatgctgga	gatctcctgg	gaggcgttgg	agcgtgcggg	tttcgacccg	tcgagcctgc
34081	gcggcagcgc	cggtggcgtc	ttcaccggtg	tcggtgcggt	ggactacgga	cccaggccgg
34141	acgaggcacc	cgaggaggtg	ctcggctacg	tcggcatcgg	caccgcctcc	agcgtcgcct
34201	ccggacgggt	ggcgtacacc	ctggggttgg	agggtccagc	cgtcaccgtc	gacaccgcct
34261	gctcctccgg	gctcaccgcg	gtgcacctgg	cgatggagtc	gctgcgccgc	gacgagtgca
34321	ccctggtcct	cgccggtggg	gtcaccgtga	tgagcagccc	gggtgcgttc	accgagttcc
34381	gcagccaggg	cgggttggcc	gaggacggcc	gctgcaaacc	gttctcccgc	gccgccgacg
34441	gcttcgggct	cgccgagggg	gccggggtcc	tggtgctcca	acggctgtcc	gtcgcccggg
34501	ccgagggccg	gccggtgctg	gccgtactgc	gtggctcggc	gatcaaccag	gacggtgcca

FIG. 7-48

```
34561 gcaacgggct caccgcgccg agcggccccg cccagcggcg ggtgatcagg caggcgttgg
34621 agcgggcgcg gctgcgtccc gtcgacgtgg actacgtgga ggcccacggc accggcaccc
34681 ggctgggcga tccgatcgag gcgcacgccc tgctcgacac gtacggtgcc gaccgggaac
34741 ccggccgccc gctctgggtc ggatcggtga agtccaacat cggtcacacc caggcggcgg
34801 cgggggtggc cggggtgatg aagaccgtgc tggcgctgcg gcatcgggag atcccggcga
34861 cgttgcactt cgacgagccc tcgccgcacg tcgactggga ccgggtgcg gtgtcggtgg
34921 tgtccgagac ccggccctgg ccggtggggg agcgcccgcg ccgggcgggg gtgtcctcgt
34981 tcggcatcag cggcaccaac gcgcacgtca tcgtcgagga ggcgccgagc ccgcaggcgg
35041 ccgacctcga cccgaccccc ggcccggcaa ccggagcgac ccccggaacg gatgccgccc
35101 ccaccgccga gccgggtgcg gaggcggtcg cactggtgtt ctccgcgcgc gacgagcggg
35161 ccctgcgcgc ccaggcggcc cggctcgccg accgtctcac cgacgacccg gccccctcgt
35221 tgcgcgacac cgccttcacc ctggtcaccc gccgtgccac ctgggagcat cgggcggtcg
35281 tcgtcggcgg gggcgaggag gtcctcgccg gcctccgggc cgtcgccggg ggacgtcccg
35341 tcgacggagc cgtcagcggg cgggcgcgcg ccggccgccg ggtggtgctg gtcttccccg
35401 ggcagggcgc acagtggcag ggcatggccc gggacctgct gcggcagtcg ccgaccttcg
35461 cggagtccat cgacgcctgc gagcgggcgc tcgccccgca cgtggactgg tcgctgcgcg
35521 aggtgctcga cggcgagcag tcgttggacc ccgtcgacgt ggtgcagccg gtgctgttcg
35581 cggtgatggt gtcgttggcg cggttgtggc agtcgtacgg ggtgactccg ggtgcggtgg
35641 tgggtcactc gcagggggag atcgccgccg cgcacgtggc tggtgcgttg tcgttggccg
35701 acgccgccag ggtggtggcg ttgcgcagcc gggtgctgcg ccgtctcggt ggtcacggcg
35761 ggatggcgtc gttcgggctc caccccgacc aggccgccga gcggatcgcg cgcttcgcgg
35821 gtgcgctgac tgtcgcctcg gtcaacggtc cccgttcggt ggtgctggcc ggggagaacg
35881 gcccgttgga cgagctgatc gccgagtgcg aggccgaggg cgtgaccgcc cgtcggatcc
```

FIG. 7-49

```
35941 ccgtcgacta cgcctcacac tccccgcagg tggagtcgct gcgtgaggag ctgctcgccg
36001 cactggccgg ggtccgtccg gtgtcggccg ggatccccct gtactcgacc ctgaccggtc
36061 aggtcatcga aacggcgacg atggacgccg actactggtt cgccaacctc cgggagccgg
36121 tgcgcttcca ggacgccacc aggcagctcg ccgaggcggg gttcgacgcc ttcgtcgagg
36181 tcagcccgca cccggtgttg acagtcggtg tcgaggccac cctcgaggca gtgctgcccc
36241 ccgacgcgga tccgtgtgtc acaggcaccc tgcgccgcga acgcggcggt ctcgcgcagt
36301 tccacaccgc gctcgccgag gcgtacaccc ggggggtgga ggtcgactgg cgtaccgcag
36361 tgggtgaggg acgcccggtc gacctgccgg tctacccgtt ccaacgacag aacttctggc
36421 tcccggtccc cctgggccgg gtccccgaca ccggcgacga gtggcgttac cagctcgcct
36481 ggcaccccgt cgacctcggg cggtcctccc tggccggacg ggtcctggtg gtgaccggag
36541 cggcagtacc cccggcctgg acggacgtgg tccgcgacgg cctggaacag cgcggggcga
36601 ccgtcgtgtt gtgcaccgcg cagtcgcgcg cccggatcgg cgccgcactc gacgccgtcg
36661 acggcaccgc cctgtccact gtggtctctc tgctcgcgct cgccgagggc ggtgctgtcg
36721 acgaccccag cctggacacc ctcgcgttgg tccaggcgct cggcgcagcc gggatcgacg
36781 tccccctgtg gctggtgacc agggacgccg ccgccgtgac cgtcggagac gacgtcgatc
36841 cggcccaggc catggtcggt gggctcggcc gggtggtggg cgtggagtcc cccgcccggt
36901 ggggtggcct ggtggacctg cgcgaggccg acgccgactc ggcccggtcg ctggccgcca
36961 tactggccga cccgcgcggc gaggagcagt tcgcgatccg gcccgacggc gtcaccgtcg
37021 cccgtctcgt cccggcaccg gcccgcgcgg cgggtacccg gtggacgccg cgcgggaccg
37081 tcctggtcac cggcggcacc ggcggcatcg gcgcgcacct ggcccgctgg ctcgccggtg
37141 cgggcgccga gcacctggtg ctgctcaaca ggcggggagc ggaggcggcc ggtgccgccg
37201 acctgcgtga cgaactggtc gcgctcggca cgggagtcac catcacggcc tgcgacgtcg
37261 ccgaccgcga ccggttggcg gccgtcctcg acgccgcacg ggcgcaggga cgggtggtca
```

FIG. 7-50

37321 cggcggtgtt ccacgccgcc gggatctccc ggtccacagc ggtacaggag ctgaccgaga
37381 gcgagttcac cgagatcacc gacgcgaagg tgcggggtac ggcgaacctg gccgaactct
37441 gtcccgagct ggacgccctc gtgctgttct cctcgaacgc ggcggtgtgg ggcagcccgg
37501 ggctggcctc ctacgcggcg ggcaacgcct tcctcgacgc cttcgcccgt cgtggtcggc
37561 gcagtgggct gccggtcacc tcgatcgcct ggggtctgtg ggccgggcag aacatggccg
37621 gtaccgaggg cggcgactac ctgcgcagcc agggcctgcg cgccatggac ccgcagcggg
37681 cgatcgagga gctgcggacc accctggacg ccggggaccc gtgggtgtcg gtggtggacc
37741 tggaccggga gcggttcgtc gaactgttca ccgccgcccg ccgccggccc ctcttcgacg
37801 aactcggtgg ggtccgcgcc ggggccgagg agaccggtca ggaatcggat ctcgcccggc
37861 ggctggcgtc gatgccggag gccgaacgtc acgagcatgt cgcccggctg gtccgagccg
37921 aggtggcagc ggtgctgggc cacggcacgc cgacggtgat cgagcgtgac gtcgccttcc
37981 gtgacctggg attcgactcc atgaccgccg tcgacctgcg gaaccggctc gcggcggtga
38041 ccggggtccg ggtggccacg accatcgtct tcgaccaccc gacagtggac cgcctcaccg
38101 cgcactacct ggaacgactc gtcggtgagc cggaggcgac gaccccggct gcggcggtcg
38161 tcccgcaggc acccggggag gccgacgagc cgatcgcgat cgtcgggatg gcctgccgcc
38221 tcgccggtgg agtgcgtacc cccgaccagt tgtgggactt catcgtcgcc gacggcgacg
38281 cggtcaccga gatgccgtcg gaccggtcct gggacctcga cgcgctgttc gacccggacc
38341 ccgagcggca cggcaccagc tactcccggc acggcgcgtt cctggacggg gcggccgact
38401 tcgacgcggc gttcttcggg atctcgccgc gtgaggcgtt ggcgatggat ccgcagcagc
38461 ggcaggtcct ggagacgacg tgggagctgt tcgagaacgc cggcatcgac ccgcactccc
38521 tgcgcggtac ggacaccggt gtcttcctcg gcgctgcgta ccaggggtac ggccagaacg
38581 cgcaggtgcc gaaggagagt gagggttacc tgctcaccgg tggttcctcg gcggtcgcct
38641 ccggtcggat cgcgtacgtg ttggggttgg aggggccggc gatcactgtg gacacggcgt

FIG. 7-51

38701 gttcgtcgtc gcttgtggcg ttgcacgtgg cggccgggtc gctgcgatcg ggtgactgtg
38761 ggctcgcggt ggcgggtggg gtgtcggtga tggccggtcc ggaggtgttc accgagttct
38821 ccaggcaggg cgcgctggcc cccgacggtc ggtgcaagcc cttctccgac caggccgacg
38881 ggttcggatt cgccgagggc gtcgctgtgg tgctcctgca gcggttgtcg gtggcggtgc
38941 gggaggggcg tcgggtgttg ggtgtggtgg tgggttcggc ggtgaatcag gatggggcga
39001 gtaatgggtt ggcggcgccg tcgggggtgg cgcagcagcg ggtgattcgg cgggcgtggg
39061 gtcgtgcggg tgtgtcgggt ggggatgtgg gtgtggtgga ggcgcatggg acggggacgc
39121 ggttggggga tccggtggag ttgggggcgt tgttggggac gtatggggtg ggtcggggtg
39181 gggtgggtcc ggtggtggtg ggttcggtga aggcgaatgt gggtcatgtg caggcggcgg
39241 cgggtgtggt gggtgtgatc aaggtggtgt tggggttggg tcgggggttg gtgggtccga
39301 tggtgtgtcg gggtgggttg tcggggttgg tggattggtc gtcgggtggg ttggtggtgg
39361 cggatggggt gcgggggtgg ccggtgggtg tggatggggt gcgtcggggt ggggtgtcgg
39421 cgtttggggt gtcggggacg aatgctcatg tggtggtggc ggaggcgccg gggtcggtgg
39481 tggggcggga acggccggtg gaggggtcgt cgcgggggtt ggtgggggtg gctggtggtg
39541 tggtgccggt ggtgctgtcg gcaaagaccg aaaccgccct gaccgagctc gcccgacgac
39601 tgcacgacgc cgtcgacgac accgtcgccc tcccggcggt ggccgccacc ctcgccaccg
39661 gacgcgccca cctgccctac cgggccgccc tgctggcccg cgaccacgac gaactgcgcg
39721 acaggctgcg ggcgttcacc actggttcgg cggctcccgg tgtggtgtcg ggggtggcgt
39781 cgggtggtgg tgtggtgttt gtttttcctg gtcagggtgg tcagtgggtg gggatggcgc
39841 gggggttgtt gtcggttccg gtgtttgtgg agtcggtggt ggagtgtgat gcggtggtgt
39901 cgtcggtggt ggggttttcg gtgttggggg tgttggaggg tcggtcgggt gcgccgtcgt
39961 tggatcgggt ggatgtggtg cagccggtgt tgttcgtggt gatggtgtcg ttggcgcggt
40021 tgtggcggtg gtgtggggtt gtgcctgcgg cggtggtggg tcattcgcag ggggagatcg

FIG. 7-52

40081 cggcggcggt ggtggcgggg gtgttgtcgg tgggtgatgg tgcgcgggtg gtggcgttgc
40141 gggcgcgggc gttgcgggcg ttggccggcc acggcggcat ggtctccctc gcggtctccg
40201 ccgaacgcgc ccgggagctg atcgcaccct ggtccgaccg gatctcggtg gcggcggtca
40261 actccccgac ctcggtggtg gtctcgggtg acccacaggc cctcgccgcc ctcgtcgccc
40321 actgcgccga gaccggtgag cgggccaaga cgctgcctgt ggactacgcc tcccactccg
40381 cccacgtcga acagatccgc gacacgatcc tcaccgacct ggccgacgtc acggcgcgcc
40441 gacccgacgt cgccctctac tccacgctgc acggcgcccg gggcgccggc acggacatgg
40501 acgcccggta ctggtacgac aacctgcgct caccggtgcg cttcgacgag gccgtcgagg
40561 ccgccgtcgc cgacggctac cgggtcttcg tcgagatgag cccacacccg gtcctcaccg
40621 ccgcggtgca ggagatcgac gacgagacgg tggccatcgg ctcgctgcac cgggacaccg
40681 gcgagcggca cctggtcgcc gaactcgccc gggcccacgt gcacggcgta ccagtggact
40741 ggcgggcgat cctccccgcc acccacccgg ttcccctgcc gaactacccg ttcgaggcga
40801 cccggtactg gctcgccccg acggcggccg accaggtcgc cgaccaccgc taccgcgtcg
40861 actggcggcc cctggccacc accccggcgg agctgtccgg cagctacctc gtcttcggcg
40921 acgccccgga gaccctcggc cacagcgtcg agaaggccgg cgggctcctc gtcccggtgg
40981 ccgctcccga ccgggagtcc ctcgcggtcg ccctggacga ggcggccgga cgactcgccg
41041 gtgtgctctc cttcgccgcc gacaccgcca cccacctggc ccggcaccga ctcctcggcg
41101 aggccgacgt cgaggcccca ctctggctgg tcaccagcgg cggcgtcgca ctcgacgacc
41161 acgacccgat cgactgcgac caggcaatgg tgtgggggat cggacgggtg atgggtctgg
41221 agaccccgca ccggtggggc ggcctggtgg acgtgaccgt cgaacccacc gccgaggacg
41281 gggtggtctt cgccgccctc ctggccgccg acgaccacga ggaccaggtg gcgctgcgcg
41341 acggcatccg ccacggccga cggctcgtcc gcgccccgct gaccacccga aacgccaggt
41401 ggacaccggc gggcacggcg ctcgtcacgg gcggtacggg tgccctcggc ggccacgtcg

FIG. 7-53

```
41461 cgcggtacct ggcccggtcc ggggtgaccg atctcgtcct gctcagcagg agcggccccg
41521 acgcacccgg tgccgccgaa ctggccgccg aactggccga cctcggggcc gagccgagag
41581 tcgaggcgtg cgacgtcacc gacgggccac gcctgcgcgc cctggtgcag gagctacggg
41641 aacaggaccg gccggtccgg atcgtcgtcc acaccgcagg ggtgcccgac tcccgtcccc
41701 tcgaccggat cgacgaactg gagtcggtca gcgccgcgaa ggtgaccggg gcgcggctgc
41761 tcgacgagct ctgcccggac gccgacacct tcgtcctgtt ctcctcgggg gcgggagtgt
41821 ggggtagcgc gaacctgggc gcgtacgcgg cagccaacgc ctacctggac gccctggccc
41881 accgccgccg ccaggcgggc cgggccgcga cctcggtcgc ctggggggcg tgggccggcg
41941 acggcatggc caccggcgac ctcgacgggc tgacccggcg cggtctgcgg gcgatggcac
42001 cggaccgggc gctgcgcgcc tgcaccaggc gttggaccac ccacgacacc tgtgtgtcgg
42061 tagccgacgt cgactgggac cgcttcgccg tgggtttcac cgccgcccgg cccagacccc
42121 tgatcgacga actcgtcacc tccgcgccgg tggccgcccc caccgctgcg gcggccccgg
42181 tcccggcgat gaccgccgac cagctactcc agttcacgcg ctcgcacgtg gccgcgatcc
42241 tcggtcacca ggacccggac gcggtcgggt tggaccagcc cttcaccgag ctgggcttcg
42301 actcgctcac cgccgtcggc ctgcgcaacc agctccagca ggccaccggg cggacgctgc
42361 ccgccgccct ggtgttccag caccccacgg tacgcagact cgccgaccac ctcgcgcagc
42421 agctcgacgt cggcaccgcc ccggtcgagg cgacgggcag cgtcctgcgg gacggctacc
42481 ggcgggccgg gcagaccggc gacgtccggt cgtacctgga cctgctggcg aacctgtcgg
42541 agttccggga gcggttcacc gacgcggcga gcctgggcgg acagctggaa ctcgtcgacc
42601 tggccgacgg atccggcccg gtcactgtga tctgttgcgc gggcactgcg gcgctctccg
42661 ggccgcacga gttcgcccga ctcgcctcgg cgctgcgcgg caccgtgccg gtgcgcgccc
42721 tcgcgcaacc cgggtacgag gcgggtgaac cggtgccggc gtcgatggag gcagtgctcg
42781 gggtgcaggc ggacgcggtc ctcgcggcac agggcgacac gccgttcgtg ctggtcggac
```

FIG. 7-54

```
42841 actcggcggg ggccctgatg gcgtacgccc tggcgaccga gctggccgac cggggccacc
42901 cgccacgtgg cgtcgtgctc ctcgacgtgt acccacccgg tcaccaggag gcggtgcacg
42961 cctggctcgg cgagctgacc gccgccctgt tcgaccacga gaccgtacgg atggacgaca
43021 cccggctcac ggccctgggg gcgtacgaca ggctgaccgg caggtggcgt ccgagggaca
43081 ccggtctgcc cacgctggtg gtggccgcca gcgagccgat gggggagtgg ccggacgacg
43141 gttggcagtc cacgtggccg ttcgggcacg acagggtcac ggtgcccggt gaccacttct
43201 cgatggtgca ggagcacgcc gacgcgatcg cgcggcacat cgacgcctgg ttgagcgggg
43261 agagggcatg aacacgaccg atcgcgccgt gctgggccga cgactccaga tgatccgggg
43321 actgtactgg ggttacggca gcaacggaga cccgtacccg atgctgttgt gcgggcacga
43381 cgacgacccg caccgctggt accgggggct gggcggatcc ggggtccggc gcagccgtac
43441 cgagacgtgg gtggtgaccg accacgccac cgccgtgcgg gtgctcgacg acccgacctt
43501 cacccgggcc accggccgga cgccggagtg gatgcgggcc gcgggcgccc cggcctcgac
43561 ctgggcgcag ccgttccgtg acgtgcacgc cgcgtcctgg gacgccgaac tgcccgaccc
43621 gcaggaggtg gaggaccggc tgacgggtct cctgcctgcc ccggggaccc gcctggacct
43681 ggtccgcgac ctcgcctggc cgatggcgtc gcgggggtc ggcgcggacg accccgacgt
43741 gctgcgcgcc gcgtgggacg cccgggtcgg cctcgacgcc cagctcaccc cgcagcccct
43801 ggcggtgacc gaggcggcga tcgccgcggt gcccggggac ccgcaccggc gggcgctgtt
43861 caccgccgtc gagatgacag ccaccgcgtt cgtcgacgcg gtgctggcgg tgaccgccac
43921 ggcgggggcg gcccagcgtc tcgccgacga ccccgacgtc gccgcccgtc tcgtcgcgga
43981 ggtgctgcgc ctgcatccga cggcgcacct ggaacggcgt accgccggca ccgagacggt
44041 ggtgggcgag cacacggtcg cggcgggcga cgaggtcgtc gtggtggtcg ccgccgccaa
44101 ccgtgacgcg gggtcttcg ccgacccgga ccgcctcgac ccggaccggg ccgacgccga
44161 ccgggccctg tccgcccagc gcggtcaccc cggccggttg gaggagctgg tggtggtcct
```

FIG. 7-55

```
44221 gaccaccgcc gcactgcgca gcgtcgccaa ggcgctgccc ggtctcaccg ccggtggccc
44281 ggtcgtcagg cgacgtcgtt caccggtcct gcgagccacc gcccactgcc cggtcgaact
44341 ctgaggtgcc tgcgatgcgc gtcgtcttct cctccatggc cagcaagagc cacctgttcg
44401 gtctcgttcc cctcgcctgg gccttccgcg cggcgggcca cgaggtacgg gtcgtcgcct
44461 caccggctct caccgacgac atcacggcgg ccggactgac ggccgtaccg gtcggcaccg
44521 acgtcgacct tgtcgacttc atgacccacg ccgggtacga catcatcgac tacgtccgca
44581 gcctggactt cagcgagcgg gacccggcca cctccacctg ggaccacctg ctcggcatgc
44641 agaccgtcct caccccgacc ttctacgccc tgatgagccc ggactcgctg gtcgagggca
44701 tgatctcctt ctgtcggtcg tggcgacccg actggtcgtc tggaccgcag accttcgccg
44761 cgtcgatcgc ggcgacggtg accggcgtgg cccacgcccg actcctgtgg ggacccgaca
44821 tcacggtacg ggcccggcag aagttcctcg ggctgctgcc cggacagccc gccgcccacc
44881 gggaggaccc cctcgccgag tggctcacct ggtctgtgga gaggttcggc ggccgggtgc
44941 cgcaggacgt cgaggagctg gtggtcgggc agtggacgat cgaccccgcc ccggtcggga
45001 tgcgcctcga caccgggctg aggacggtgg gcatgcgcta cgtcgactac aacggcccgt
45061 cggtggtgcc ggactggctg cacgacgagc cgacccgccg acgggtctgc ctcaccctgg
45121 gcatctccag ccgggagaac agcatcgggc aggtctccgt cgacgacctg ttgggtgcgc
45181 tcggtgacgt cgacgccgag atcatcgcga cagtggacga gcagcagctc gaaggcgtcg
45241 cccacgtccc ggccaacatc cgtacggtcg ggttcgtccc gatgcacgca ctgctgccga
45301 cctgcgcggc gacggtgcac cacggcggtc ccggcagctg gcacaccgcc gccatccacg
45361 gcgtgccgca ggtgatcctg cccgacggct gggacaccgg ggtccgcgcc cagcggaccg
45421 aggaccaggg ggcgggcatc gccctgccgg tgcccgagct gacctccgac cagctccgcg
45481 aggcggtgcg gcgggtcctg gacgatcccg ccttcaccgc cggtgcggcg cggatgcggg
45541 ccgacatgct cgccgagccg tcccccgccg aggtcgtcga cgtctgtgcg gggctggtcg
```

FIG. 7-56

```
45601 gggaacggac cgccgtcgga tgagcaccga cgccacccac gtccggctcg gccggtgcgc
45661 cctgctgacc agccggctct ggctgggtac ggcagccctc gccggccagg acgacgccga
45721 cgcagtacgc ctgctcgacc acgcccgttc ccggggcgtc aactgcctcg acaccgccga
45781 cgacgactct gcgtcgacca gtgcccaggt cgccgaggag tcggtcggcc ggtggttggc
45841 cggggacacc ggtcggcggg aggagaccgt cctgtcggtg acggtgggtg tcccaccggg
45901 cgggcaggtc ggcgggggcg gcctctccgc ccggcagatc atcgcctcct gtgagggctc
45961 cctgcggcgt ctcggtgtcg accacgtcga cgtccttcac ctgccccggg tggaccgggt
46021 ggagccgtgg gacgaggtct ggcaggcggt ggacgccctc gtggccgccg gaaaggtctg
46081 ttacgtcggg tcgtcgggct tccccggatg gcacatcgtc gccgcccagg agcacgccgt
46141 ccgccgtcac cgcctcggcc tggtgtccca ccagtgtcgg tacgacctga cgtcgcgcca
46201 tcccgaactg gaggtcctgc ccgccgcgca ggcgtacggg ctcggggtct tcgccaggcc
46261 gacccgcctc ggcggtctgc tcggcggcga cggtccgggc gccgcagccg cacgggcgtc
46321 gggacagccg acggcactgc gctcggcggt ggaggcgtac gaggtgttct gcagagacct
46381 cggcgagcac cccgccgagg tcgcactggc gtgggtgctg tcccggcccg gtgtggcggg
46441 ggcggtcgtc ggtgcgcgga cgcccggacg gctcgactcc gcgctccgcg cctgcggcgt
46501 cgccctcggc gcgacggaac tcaccgccct ggacgggatc ttccccgggg tcgccgcagc
46561 aggggcggcc ccggaggcgt ggctacggtg agagcccgcc cctgacctgc gggaacccgt
46621 gtcggtgcgg cgggacggcc gccgcggtcc ccgccccggt cagccggtgg gggtgagccg
46681 cagcaggtcc ggcgccaccg actcggccac ctccccgacg tggtcggcga ggtagaagtg
46741 cccgcccggg aaggtccggg tacggccggg gactaccgag tacggcagcc agcgttgggc
46801 gtcctccacc gtcgtcaacg ggtcggtgtc accgcagagg gtggtgatgc cggcccgcag
46861 cggcggcccg gcctgccagg cgtaggagcg cagcacccgg tggtcggccc gcagcaccgg
46921 cagcgacatg tccaacagcc cctggtcggc caatgcggcc tcgctgaccc cgagcctgcg
```

FIG. 7-57

```
46981 catctgctcg acgagtccgt cctcgtcggg caggtcggtg cgccgctcgt ggacccgggg
47041 ggcggtctgc ccggagacga acaaccgcag cggtcgcacc cccggacgag cctccaggcg
47101 acgggcggtc tcgtaggcga ccagggcgcc catgctgtga ccgaacaggg cgaacggaac
47161 ctcgccgacg aggtcgcgca gcacggccgc gacctcgtcg gcgatctccc cggcggtgcc
47221 gagagcccgc tcgtcacgtc ggtcctgccg gcccgggtac tgcaccgccc acacgtcgac
47281 ctccggggcc agtgcccggg cgaggtcgag gtacgagtcg gcggcggctc ccgcgtgcgg
47341 gaagcagtac agccgggccc ggtgtccgtc ggcggacccg aaccgccgca accaggtgtt
47401 catcggtgtc tcatccgttc ggtcgcaccg gcaggtggtc gatgccgcgc agcaggagcg
47461 accgccgcca gacaacctcg tcggagggga agcccagcga cagcttcggg aagcggtcga
47521 acagggcccc cagggcgacc tctccctcca gcttggccag cgggcggccc atgcagtagt
47581 ggatgccgtg cccgaaggtg aggtgtcccc ggctgtccct ggtgacgtcg aaccggtcgg
47641 ggtcggggaa ctgtcccggg tcgcggttgg ccgccccgtt ggcgatcagg acggtgctgt
47701 acgccgggat cgtcaccccg ccgatctcca cctcggcggt ggcgaaccgg gtggtggtct
47761 ccggtggggc ctggtagcgc aggatctcct ccaccgctcc gggcagcagt gccgggtcct
47821 tccggaccag cgcgagctgg tcggggtggg tcagcagcag gtaggtgccg atcccgatga
47881 ggctcaccga cgcctcgaat cccgccagca gcagcaccag cgcgatggat gtgagttcgt
47941 cgcggctgag ccggtcggcg tcgtcgtcct ggacccggat c
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(SEQ ID NO: 1)

FIG. 7-58

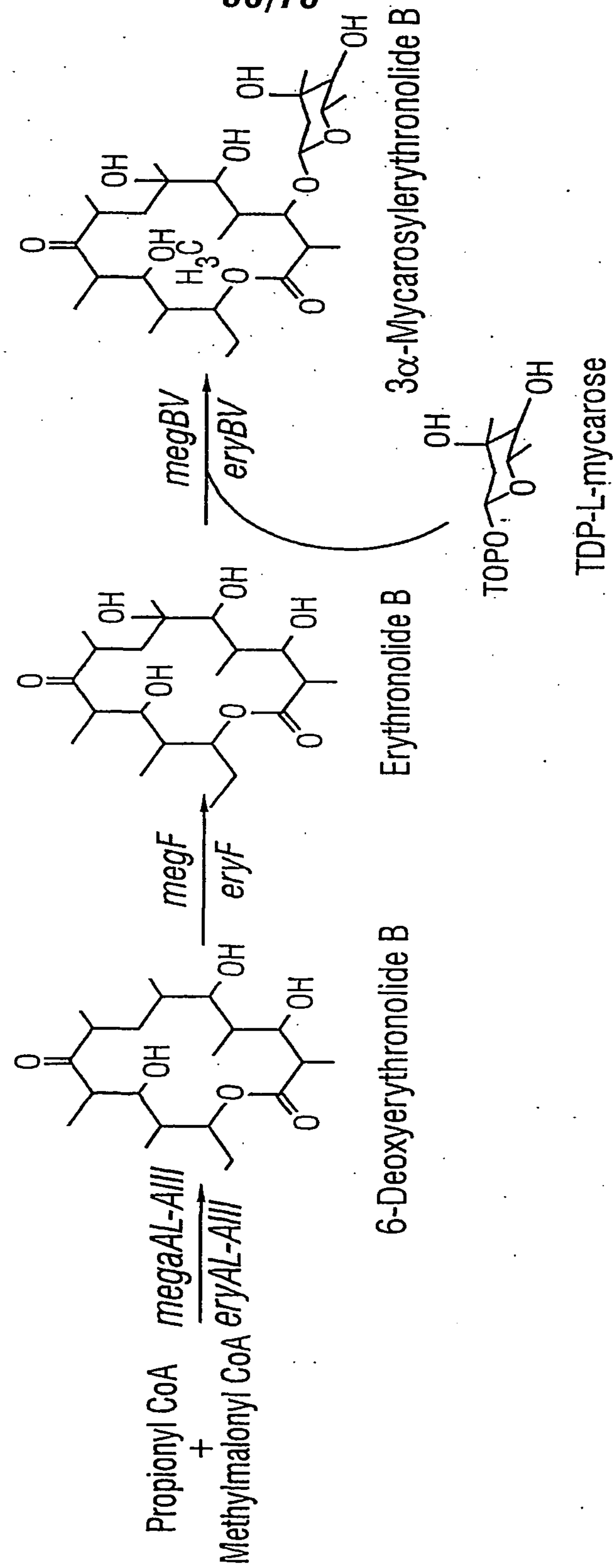
Propionyl CoA
+
Methylmalonyl CoA
megaAL-AIII
eryAL-AIII
6-Deoxyerythronolide B
megF
eryF
Erythronolide B
megBV
eryBV
TDP-L-mycarose
TOPO
3α-Mycarosylerythronolide B
H_3C
OH
O

FIG. 8A

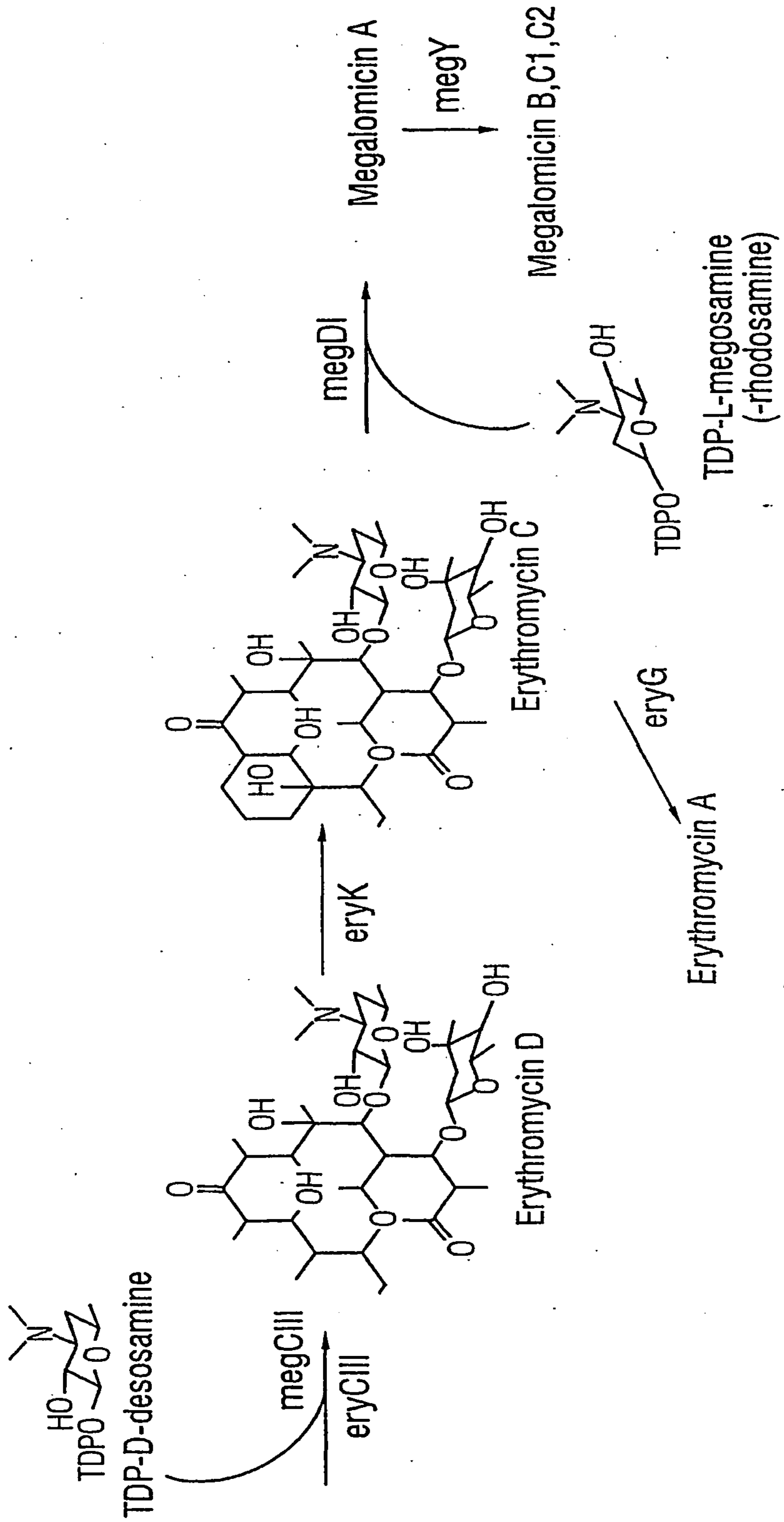
TDP-D-desosamine
megCIII
eryCIII
Erythromycin D
eryK
Erythromycin C
eryG
Erythromycin A
megDI
TDP-L-megosamine
(-rhodosamine)
Megalomicin A
megY
Megalomicin B,C1,C2

FIG. 8B

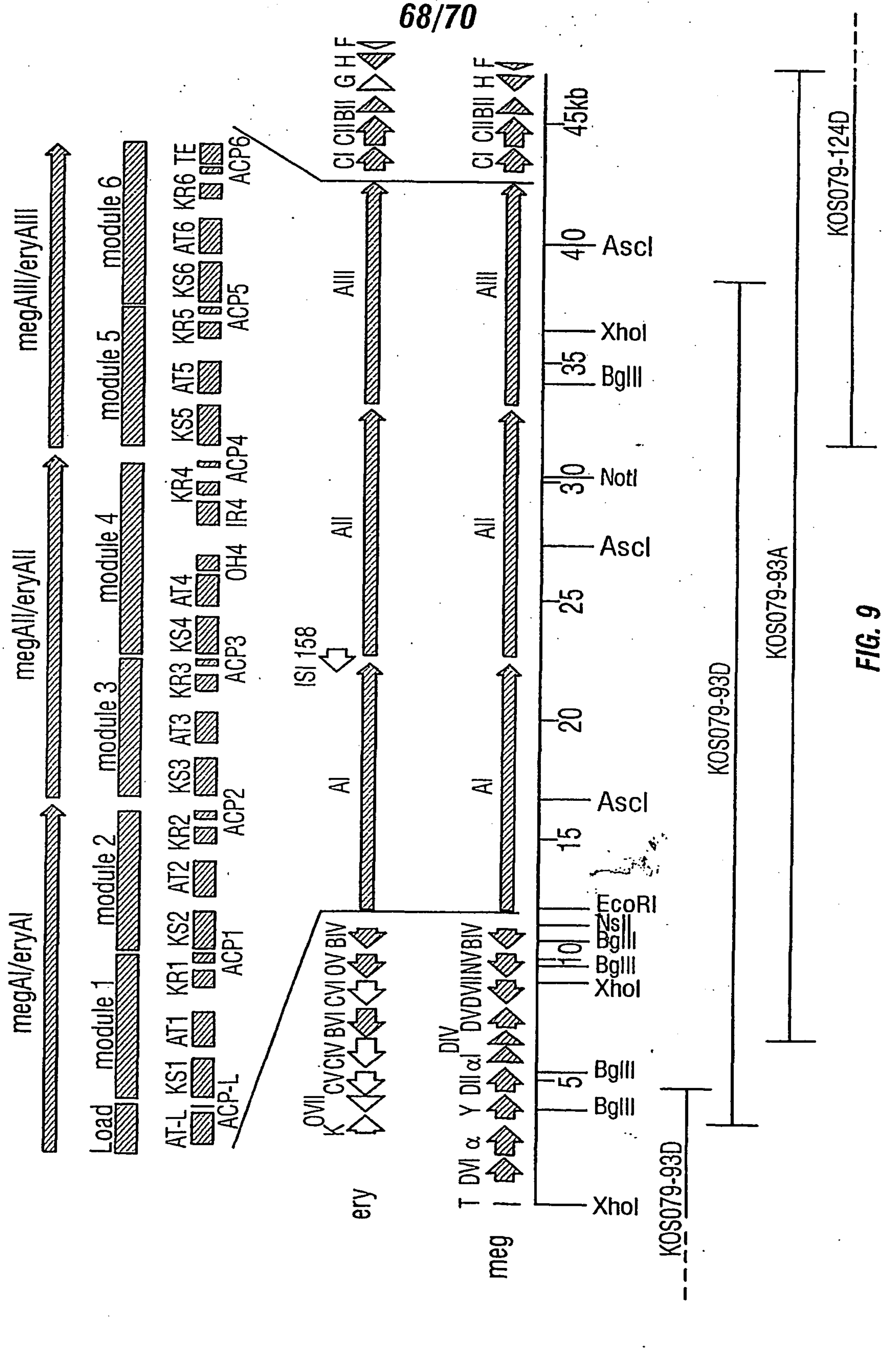
megAI/eryAI
megAII/eryAII
megAIII/eryAIII
Load
module 1
module 2
module 3
module 4
module 5
module 6
AT-L KS1 AT1 KR1 KS2 AT2 KR2 KS3 AT3 KR3 KS4 AT4 KR4 KS5 AT5 KR5 KS6 AT6 KR6 TE
ACP-L ACP1 ACP2 ACP3 OH4 IR4 ACP4 ACP5 ACP6
ISI 158
ery
K OVII CVCIV BVI CVI OV BIV
AI AII AIII
CI CIIBII G H F
meg
T DVI α Y DII α DIV DVDVIINVBIV
CI CIIBII H F
XhoI BglII BglII XhoI BglII BglII NsiI EcoRI AscI AscI NotI BglII XhoI AscI
5 10 15 20 25 30 35 40 45kb
KOS079-93D
KOS079-93D
KOS079-93A
KOS079-124D

FIG. 9

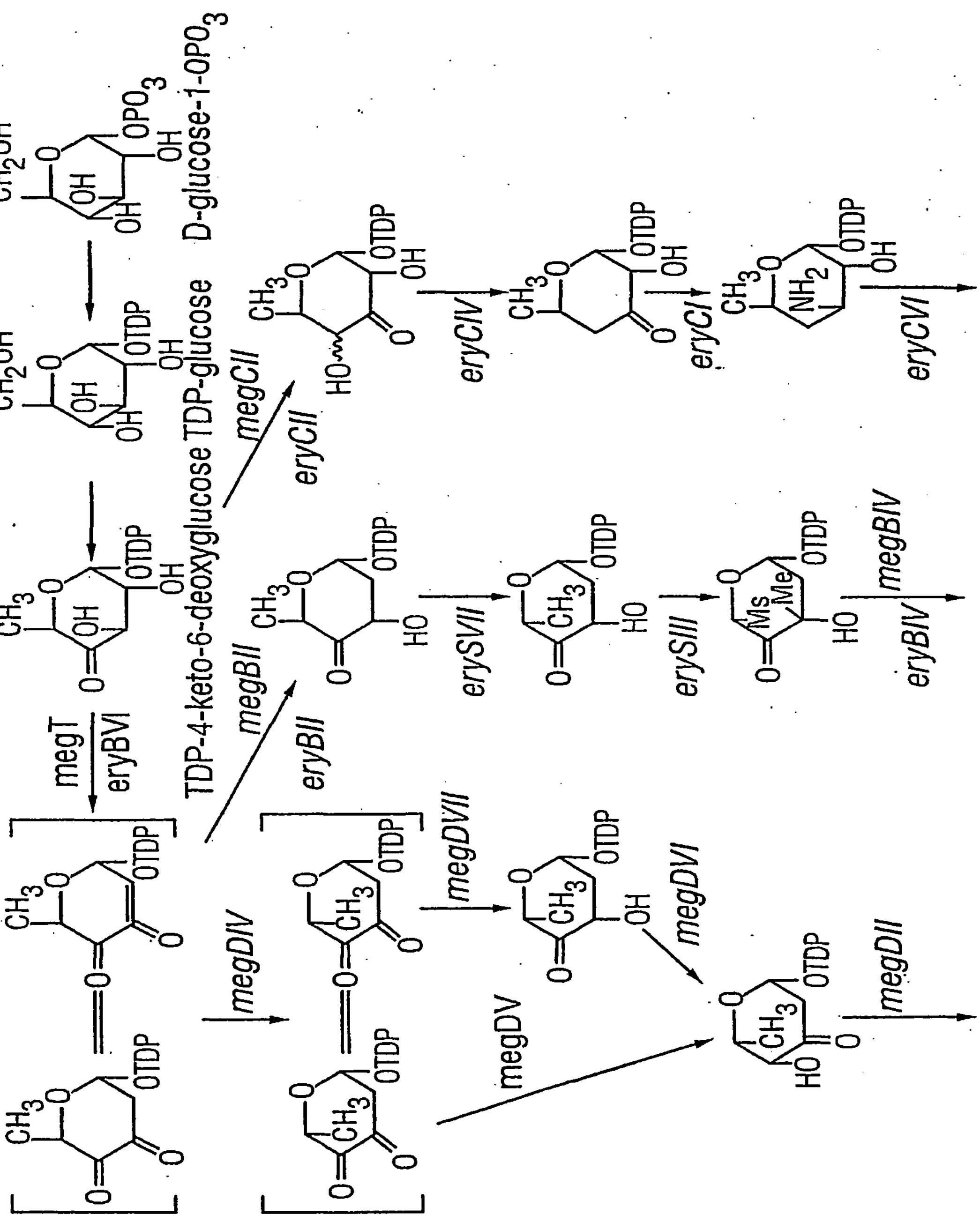
D-glucose-1-OPO3
TDP-glucose
TDP-4-keto-6-deoxyglucose
megT
eryBVI
megCII
eryCII
eryCIV
eryCI
eryCVI
megBII
eryBII
erySVII
erySIII
megBIV
eryBIV
megDIV
megDVII
megDVI
megDV
megDII

FIG. 10A

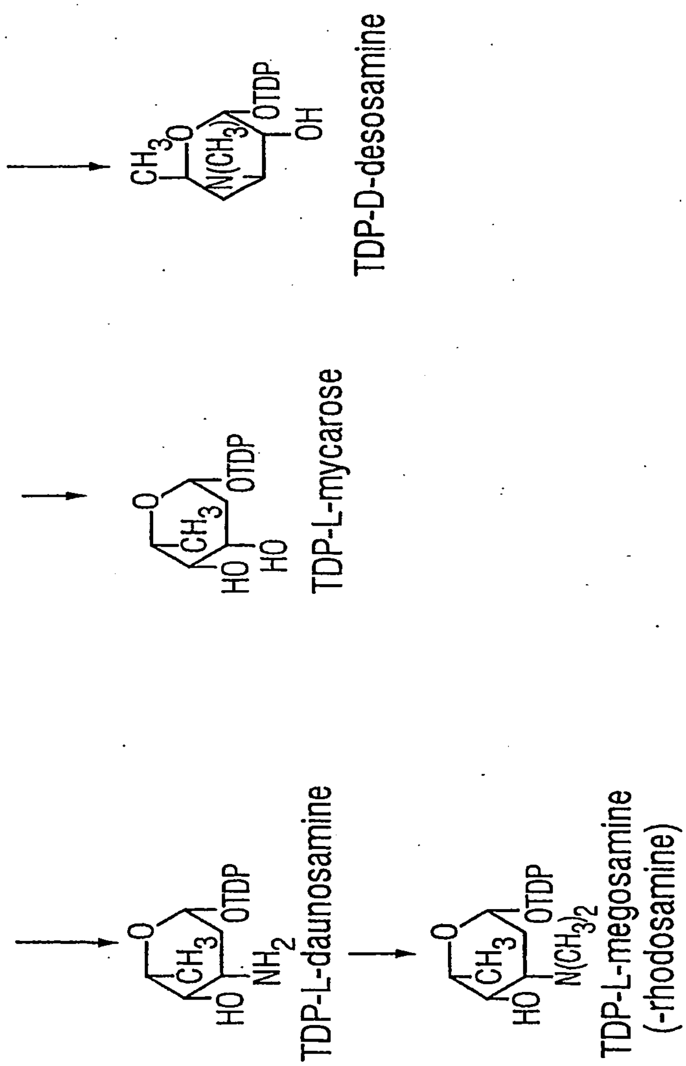
TDP-D-desosamine
TDP-L-mycarose
TDP-L-daunosamine
TDP-L-megosamine
(-rhodosamine)

FIG. 10B

SEQUENCE LISTING

<110> Kosan Biosciences, Inc.

<120> Recombinant Megalomicin Biosynthetic
Genes and Uses Thereof

<130> 300622004740

<140> To be assigned
<141> Herewith

<150> US 60/158,305
<151> 1999-10-08

<150> US 60/190,024
<151> 2000-03-17

<160> 34

<170> FastSEQ for Windows Version 4.0

<210> 1
<211> 47981
<212> DNA
<213> Micromonospora megalomicea

<220>
<221> CDS
<222> (1)...(144)
<223> megBVI(megT), TDP-4-keto-6-deoxyglucose-2,3-dehydratase;
SEQ ID NO: 2= translated amino acid sequence

<221> CDS
<222> (928)...(2061)
<223> megDVI, TDP-4-keto-6-deoxyglucose 3,4-isomerase,
TDP-4-keto-6-deoxyhexose 3,4-isomerase;
SEQ ID NO: 3= translated amino acid sequence

<221> CDS
<222> (2072)...(3382)
<223> megDI, rhodosaminyl transferase (eryCIII homolog),
TDP-megosamine glycosyltransferase;
SEQ ID NO: 4= translated amino acid sequence

<221> CDS
<222> (3462)...(4634)
<223> megG(megY), mycarosyl acyltransferase, mycarose O-acyltransferase;
SEQ ID NO: 5= translated amino acid sequence

<221> CDS
<222> (4651)...(5775)
<223> megDII, deoxysugar transaminase (eryCI, DnrJ homolog),
TDP-3-keto-6-deoxyhexose 3-aminotransaminase;
SEQ ID NO: 6= translated amino acid sequence

<221> CDS
<222> (5822)...(6595)
<223> megDIII, daunosaminyl-N,N-dimethyltransferase (eryCVI homolog);
SEQ ID NO: 7= translated amino acid sequence

<221> CDS
<222> (6592)...(7197)
<223> megDIV, TDP-4-keto-6-deoxyglucose 3,5-epimerase (eryBVII, dnmU homolog), TDP-4-keto-6-deoxyhexose 3,5-epimerase;
SEQ ID NO: 8= translated amino acid sequence

<221> CDS
<222> (7220)...(8206)
<223> megDV, TDP-hexose 4-ketoreductase (eryBIV, dnmV homolog), TDP-4-keto-6-deoxyhexose 4-ketoreductase;
SEQ ID NO NO: 9= translated amino acid sequence

<221> CDS
<222> (8228)...(9220)
<223> megBII-1(megDVII), TDP-4-keto-L-6-deoxy-hexose 2,3-reductase;
SEQ ID NO: 10= translated amino acid sequence

<221> CDS
<222> (9226)...(10479)
<223> megBV, mycarosyl transferase, mycarose glycosyltransferase;
SEQ ID NO: 11= translated amino acid sequence

<221> CDS
<222> (10483)...(11424)
<223> megBIV, TDP-hexose 4-keotreductase, TDP-4-keto-6-deoxyhexose 4-ketoreductase;
SEQ ID NO: 12= translated amino acid sequence

<221> CDS
<222> (12181)...(22821)
<223> megAI; SEQ ID NO: 13= translated amino acid sequence

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<222> (12505)...(13470)
<223> megAI, AT-L

<221> misc_feature
<222> (13576)...(13791)
<223> megAI, ACP-L

<221> misc_feature
<222> (13849)...(15126)
<223> megAI, KS1

<221> misc_feature
<222> (15427)...(16476)
<223> megAI, AT1

<221> misc_feature
<222> (17155)...(17694)
<223> megAI, KR1

<221> misc_feature
<222> (17947)...(18207)
<223> megAI, ACP1

<221> misc_feature
<222> (18268)...(19548)
<223> megAI, KS2

<221> misc_feature

<222> (19876)...(20910)
<223> megAI, AT2

<221> misc_feature
<222> (21517)...(22053)
<223> megAI, KR2

<221> misc_feature
<222> (22318)...(22575)
<223> megAI, ACP2

<221> CDS
<222> (22867)...(33555)
<223> megAII; SEQ ID NO: 14= translated amino acid sequence

<221> misc_feature
<222> (22957)...(24237)
<223> megAII, KS3

<221> misc_feature
<222> (24544)...(25581)
<223> megAII, AT3

<221> misc_feature
<222> (26230)...(26733)
<223> megAII, KR3 (inactive)

<221> misc_feature
<222> (26998)...(27258)
<223> megAII, ACP3

<221> misc_feature
<222> (27393)...(28590)
<223> megAII, KS4

<221> misc_feature
<222> (28897)...(29931)
<223> megAII, AT4

<221> misc_feature
<222> (29953)...(30477)
<223> megAII, DH4

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<223> megAII, ER4

<221> misc_feature
<222> (32257)...(32799)
<223> megAII, KR4

<221> misc_feature
<222> (33052)...(33312)
<223> megAII, ACP4

<221> CDS
<222> (33666)...(43271)
<223> megAIII; SEQ ID NO: 15= translated amino acid sequence

<221> misc_feature
<222> (33780)...(35027)

<223> megAIII, KS5

<221> misc_feature
<222> (35385)...(36419)
<223> megAIII, AT5

<221> misc_feature
<222> (37068)...(37604)
<223> megAIII, KR5

<221> misc_feature
<222> (37860)...(38120)
<223> megAIII, ACP5

<221> misc_feature
<222> (38187)...(39470)
<223> megAIII, KS6

<221> misc_feature
<222> (39795)...(40811)
<223> megAIII, AT6

<221> misc_feature
<222> (41406)...(41936)
<223> megAIII, KR6

<221> misc_feature
<222> (42168)...(42425)
<223> megAIII, ACP6

<221> misc_feature
<222> (42585)...(43271)
<223> megAIII, TE

<221> CDS
<222> (43268)...(44344)
<223> megCII, TDP-4-keto-6-deoxyglucose 3,4-isomerase;
SEQ ID NO: 16= translated amino acid sequence

<221> CDS
<222> (44355)...(45623)
<223> megCIII, desosaminyl transferase, desosamine glycosyltransferase;
SEQ ID NO: 17= translated amino acid sequence

<221> CDS
<222> (45620)...(46591)
<223> megBII-2(megBII), TDP-4-keto-6-deoxy-L-glucose 2,3 dehydratase,
TDP-4-keto-6-deoxyglucose 2,3 dehydratase;
SEQ ID NO: 18= translated amino acid sequence

<221> CDS
<222> (46660)...(47403)
<223> megH, TEII; SEQ ID NO: 19= translated amino acid sequence

<221> CDS
<222> (47411)...(47980)
<223> megF, C-6 hydroxylase; SEQ ID NO: 20= translated amino acid sequence

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gccgatgaac gtcctgctgc ccacctgcgc ggccaccgtg caccacggcg gcaccggcag 3060
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<210> 2
<211> 48
<212> PRT
<213> Micromonospora megalomicea

<400> 2
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1 5 10 15
Ala Ile Arg Phe Leu Thr Arg His Gly Gly Pro Pro Thr Ala Thr Asp
20 25 30
Asp Val His Asp Trp Leu Ala His Arg Ala Ala Glu His Arg Leu Glu
35 40 45

<210> 3
<211> 377
<212> PRT
<213> Micromonospora megalomicea

<400> 3
Met Ala Val Gly Asp Arg Arg Arg Leu Gly Arg Glu Leu Gln Met Ala
1 5 10 15
Arg Gly Leu Tyr Trp Gly Phe Gly Ala Asn Gly Asp Leu Tyr Ser Met
20 25 30
Leu Leu Ser Gly Arg Asp Asp Asp Pro Trp Thr Trp Tyr Glu Arg Leu
35 40 45
Arg Ala Ala Gly Arg Gly Pro Tyr Ala Ser Arg Ala Gly Thr Trp Val
50 55 60
Val Gly Asp His Arg Thr Ala Ala Glu Val Leu Ala Asp Pro Gly Phe
65 70 75 80
Thr His Gly Pro Pro Asp Ala Ala Arg Trp Met Gln Val Ala His Cys
85 90 95
Pro Ala Ala Ser Trp Ala Gly Pro Phe Arg Glu Phe Tyr Ala Arg Thr
100 105 110
Glu Asp Ala Ala Ser Val Thr Val Asp Ala Asp Trp Leu Gln Gln Arg
115 120 125
Cys Ala Arg Leu Val Thr Glu Leu Gly Ser Arg Phe Asp Leu Val Asn
130 135 140
Asp Phe Ala Arg Glu Val Pro Val Leu Ala Leu Gly Thr Ala Pro Ala
145 150 155 160
Leu Lys Gly Val Asp Pro Asp Arg Leu Arg Ser Trp Thr Ser Ala Thr
165 170 175
Arg Val Cys Leu Asp Ala Gln Val Ser Pro Gln Gln Leu Ala Val Thr
180 185 190
Glu Gln Ala Leu Thr Ala Leu Asp Glu Ile Asp Ala Val Thr Gly Gly
195 200 205
Arg Asp Ala Ala Val Leu Val Gly Val Val Ala Glu Leu Ala Ala Asn
210 215 220
Thr Val Gly Asn Ala Val Leu Ala Val Thr Glu Leu Pro Glu Leu Ala
225 230 235 240
Ala Arg Leu Ala Asp Asp Pro Glu Thr Ala Thr Arg Val Val Thr Glu
245 250 255
Val Ser Arg Thr Ser Pro Gly Val His Leu Glu Arg Arg Thr Ala Ala
260 265 270
Ser Asp Arg Arg Val Gly Gly Val Asp Val Pro Thr Gly Gly Glu Val
275 280 285

Thr Val Val Val Ala Ala Ala Asn Arg Asp Pro Glu Val Phe Thr Asp
290 295 300
Pro Asp Arg Phe Asp Val Asp Arg Gly Gly Asp Ala Glu Ile Leu Ser
305 310 315 320
Ser Arg Pro Gly Ser Pro Arg Thr Asp Leu Asp Ala Leu Val Ala Thr
325 330 335
Leu Ala Thr Ala Ala Leu Arg Ala Ala Ala Pro Val Leu Pro Arg Leu
340 345 350
Ser Arg Ser Gly Pro Val Ile Arg Arg Arg Arg Ser Pro Val Ala Arg
355 360 365
Gly Leu Ser Arg Cys Pro Val Glu Leu
370 375

<210> 4
<211> 436
<212> PRT
<213> Micromonospora megalomicea

<400> 4
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1 5 10 15
Leu Val Pro Leu Ala Ser Ala Phe Gln Ala Ala Gly His Glu Val Arg
20 25 30
Val Val Ala Ser Pro Ala Leu Thr Asp Asp Val Thr Gly Ala Gly Leu
35 40 45
Thr Ala Val Pro Val Gly Asp Asp Val Glu Leu Val Glu Trp His Ala
50 55 60
His Ala Gly Gln Asp Ile Val Glu Tyr Met Arg Thr Leu Asp Trp Val
65 70 75 80
Asp Gln Ser His Thr Thr Met Ser Trp Asp Asp Leu Leu Gly Met Gln
85 90 95
Thr Thr Phe Thr Pro Thr Phe Phe Ala Leu Met Ser Pro Asp Ser Leu
100 105 110
Ile Asp Gly Met Val Glu Phe Cys Arg Ser Trp Arg Pro Asp Trp Ile
115 120 125
Val Trp Glu Pro Leu Thr Phe Ala Ala Pro Ile Ala Ala Arg Val Thr
130 135 140
Gly Thr Pro His Ala Arg Met Leu Trp Gly Pro Asp Val Ala Thr Arg
145 150 155 160
Ala Arg Gln Ser Phe Leu Arg Leu Leu Ala His Gln Glu Val Glu His
165 170 175
Arg Glu Asp Pro Leu Ala Glu Trp Phe Asp Trp Thr Leu Arg Arg Phe
180 185 190
Gly Asp Asp Pro His Leu Ser Phe Asp Glu Glu Leu Val Leu Gly Gln
195 200 205
Trp Thr Val Asp Pro Ile Pro Glu Pro Leu Arg Ile Asp Thr Gly Val
210 215 220
Arg Thr Val Gly Met Arg Tyr Val Pro Tyr Asn Gly Pro Ser Val Val
225 230 235 240
Pro Ala Trp Leu Leu Arg Glu Pro Glu Arg Arg Arg Val Cys Leu Thr
245 250 255
Leu Gly Gly Ser Ser Arg Glu His Gly Ile Gly Gln Val Ser Ile Gly
260 265 270
Glu Met Leu Asp Ala Ile Ala Asp Ile Asp Ala Glu Phe Val Ala Thr
275 280 285
Phe Asp Asp Gln Gln Leu Val Gly Val Gly Ser Val Pro Ala Asn Val
290 295 300
Arg Thr Ala Gly Phe Val Pro Met Asn Val Leu Leu Pro Thr Cys Ala
305 310 315 320
Ala Thr Val His His Gly Gly Thr Gly Ser Trp Leu Thr Ala Ala Ile
325 330 335

His Gly Val Pro Gln Ile Ile Leu Ser Asp Ala Asp Thr Glu Val His
340 345 350
Ala Lys Gln Leu Gln Asp Leu Gly Ala Gly Leu Ser Leu Pro Val Ala
355 360 365
Gly Met Thr Ala Glu His Leu Arg Gly Ala Ile Glu Arg Val Leu Asp
370 375 380
Glu Pro Ala Tyr Arg Leu Gly Ala Glu Arg Met Arg Asp Gly Met Arg
385 390 395 400
Thr Asp Pro Ser Pro Ala Gln Val Val Gly Ile Cys Gln Asp Leu Ala
405 410 415
Ala Asp Arg Ala Ala Arg Gly Arg Gln Pro Arg Arg Thr Ala Glu Pro
420 425 430
His Leu Pro Arg
435

<210> 5
<211> 390
<212> PRT
<213> Micromonospora megalomicea

<400> 5
Met Val Thr Ser Thr Asn Leu Asp Thr Thr Ala Arg Pro Ala Leu Asn
1 5 10 15
Ser Leu Thr Gly Met Arg Phe Val Ala Ala Phe Leu Val Phe Phe Thr
20 25 30
His Val Leu Ser Arg Leu Ile Pro Asn Ser Tyr Val Tyr Ala Asp Gly
35 40 45
Leu Asp Ala Phe Trp Gln Thr Thr Gly Arg Val Gly Val Ser Phe Phe
50 55 60
Phe Ile Leu Ser Gly Phe Val Leu Thr Trp Ser Ala Arg Ala Ser Asp
65 70 75 80
Ser Val Trp Ser Phe Trp Arg Arg Arg Val Cys Lys Leu Phe Pro Asn
85 90 95
His Leu Val Thr Ala Phe Ala Ala Val Val Leu Phe Leu Val Thr Gly
100 105 110
Gln Ala Val Ser Gly Glu Ala Leu Ile Pro Asn Leu Leu Leu Ile His
115 120 125
Ala Trp Phe Pro Ala Leu Glu Ile Ser Phe Gly Ile Asn Pro Val Ser
130 135 140
Trp Ser Leu Ala Cys Glu Ala Phe Phe Tyr Leu Cys Phe Pro Leu Phe
145 150 155 160
Leu Phe Trp Ile Ser Gly Ile Arg Pro Glu Arg Leu Trp Ala Trp Ala
165 170 175
Ala Val Val Phe Ala Ala Ile Trp Ala Val Pro Val Val Ala Asp Leu
180 185 190
Leu Leu Pro Ser Ser Pro Pro Leu Ile Pro Gly Leu Glu Tyr Ser Ala
195 200 205
Ile Gln Asp Trp Phe Leu Tyr Thr Phe Pro Ala Thr Arg Ser Leu Glu
210 215 220
Phe Ile Leu Gly Ile Ile Leu Ala Arg Ile Leu Ile Thr Gly Arg Trp
225 230 235 240
Ile Asn Val Gly Leu Leu Pro Ala Val Leu Leu Phe Pro Val Phe Phe
245 250 255
Val Ala Ser Leu Phe Leu Pro Gly Val Tyr Ala Ile Ser Ser Ser Met
260 265 270
Met Ile Leu Pro Leu Val Leu Ile Ile Ala Ser Gly Ala Thr Ala Asp
275 280 285
Leu Gln Gln Lys Arg Thr Phe Met Arg Asn Arg Val Met Val Trp Leu
290 295 300
Gly Asp Val Ser Phe Ala Leu Tyr Met Val His Phe Leu Val Ile Val
305 310 315 320

Tyr Gly Ala Asp Leu Leu Gly Phe Ser Gln Thr Glu Asp Ala Pro Leu
325 330 335
Gly Leu Ala Leu Phe Met Ile Ile Pro Phe Leu Ala Val Ser Leu Val
340 345 350
Leu Ser Trp Leu Leu Tyr Arg Phe Val Glu Leu Pro Val Met Arg Asn
355 360 365
Trp Ala Arg Pro Ala Ser Ala Arg Arg Lys Pro Ala Thr Glu Pro Glu
370 375 380
Gln Thr Pro Ser Arg Arg
385 390

<210> 6
<211> 374
<212> PRT
<213> Micromonospora megalomicea

<400> 6
Met Thr Thr Tyr Val Trp Ser Tyr Leu Leu Glu Tyr Glu Arg Glu Arg
1 5 10 15
Ala Asp Ile Leu Asp Ala Val Gln Lys Val Phe Ala Ser Gly Ser Leu
20 25 30
Ile Leu Gly Gln Ser Val Glu Asn Phe Glu Thr Glu Tyr Ala Arg Tyr
35 40 45
His Gly Ile Ala His Cys Val Gly Val Asp Asn Gly Thr Asn Ala Val
50 55 60
Lys Leu Ala Leu Glu Ser Val Gly Val Gly Arg Asp Asp Glu Val Val
65 70 75 80
Thr Val Ser Asn Thr Ala Ala Pro Thr Val Leu Ala Ile Asp Glu Ile
85 90 95
Gly Ala Arg Pro Val Phe Val Asp Val Arg Asp Glu Asp Tyr Leu Met
100 105 110
Asp Thr Asp Leu Val Glu Ala Ala Val Thr Pro Arg Thr Lys Ala Ile
115 120 125
Val Pro Val His Leu Tyr Gly Gln Cys Val Asp Met Thr Ala Leu Arg
130 135 140
Glu Leu Ala Asp Arg Arg Gly Leu Lys Leu Val Glu Asp Cys Ala Gln
145 150 155 160
Ala His Gly Ala Arg Arg Asp Gly Arg Leu Ala Gly Thr Met Ser Asp
165 170 175
Ala Ala Ala Phe Ser Phe Tyr Pro Thr Lys Val Leu Gly Ala Tyr Gly
180 185 190
Asp Gly Gly Ala Val Val Thr Asn Asp Asp Glu Thr Ala Arg Ala Leu
195 200 205
Arg Arg Leu Arg Tyr Tyr Gly Met Glu Glu Val Tyr Tyr Val Thr Arg
210 215 220
Thr Pro Gly His Asn Ser Arg Leu Asp Glu Val Gln Ala Glu Ile Leu
225 230 235 240
Arg Arg Lys Leu Thr Arg Leu Asp Ala Tyr Val Ala Gly Arg Arg Ala
245 250 255
Val Ala Gln Arg Tyr Val Asp Gly Leu Ala Asp Leu Gln Asp Ser His
260 265 270
Gly Leu Glu Leu Pro Val Val Thr Asp Gly Asn Glu His Val Phe Tyr
275 280 285
Val Tyr Val Val Arg His Pro Arg Arg Asp Glu Ile Ile Lys Arg Leu
290 295 300
Arg Asp Gly Tyr Asp Ile Ser Leu Asn Ile Ser Tyr Pro Trp Pro Val
305 310 315 320
His Thr Met Thr Gly Phe Ala His Leu Gly Val Ala Ser Gly Ser Leu
325 330 335
Pro Val Thr Glu Arg Leu Ala Gly Glu Ile Phe Ser Leu Pro Met Tyr
340 345 350

Pro Ser Leu Pro His Asp Leu Gln Asp Arg Val Ile Glu Ala Val Arg
355 360 365
Glu Val Ile Thr Gly Leu
370

<210> 7
<211> 257
<212> PRT
<213> Micromonospora megalomicea

<400> 7
Met Pro Asn Ser His Ser Thr Thr Ser Ser Thr Asp Val Ala Pro Tyr
1 5 10 15
Glu Arg Ala Asp Ile Tyr His Asp Phe Tyr His Gly Arg Gly Lys Gly
20 25 30
Tyr Arg Ala Glu Ala Asp Ala Leu Val Glu Val Ala Arg Lys His Thr
35 40 45
Pro Gln Ala Ala Thr Leu Leu Asp Val Ala Cys Gly Thr Gly Ser His
50 55 60
Leu Val Glu Leu Ala Asp Ser Phe Arg Glu Val Val Gly Val Asp Leu
65 70 75 80
Ser Ala Ala Met Leu Ala Thr Ala Ala Arg Asn Asp Pro Gly Arg Glu
85 90 95
Leu His Gln Gly Asp Met Arg Asp Phe Ser Leu Asp Arg Arg Phe Asp
100 105 110
Val Val Thr Cys Met Phe Ser Ser Thr Gly Tyr Leu Val Asp Glu Ala
115 120 125
Glu Leu Asp Arg Ala Val Ala Asn Leu Ala Gly His Leu Ala Pro Gly
130 135 140
Gly Thr Leu Val Val Glu Pro Trp Trp Phe Pro Glu Thr Phe Arg Pro
145 150 155 160
Gly Trp Val Gly Ala Asp Leu Val Thr Ser Gly Asp Arg Arg Ile Ser
165 170 175
Arg Met Ser His Thr Val Pro Ala Gly Leu Pro Asp Arg Thr Ala Ser
180 185 190
Arg Met Thr Ile His Tyr Thr Val Gly Ser Pro Glu Ala Gly Ile Glu
195 200 205
His Phe Thr Glu Val His Val Met Thr Leu Phe Ala Arg Ala Ala Tyr
210 215 220
Glu Gln Ala Phe Gln Arg Ala Gly Leu Ser Cys Ser Tyr Val Gly His
225 230 235 240
Asp Leu Phe Ser Pro Gly Leu Phe Val Gly Val Ala Ala Glu Pro Gly
245 250 255
Arg

<210> 8
<211> 201
<212> PRT
<213> Micromonospora megalomicea

<400> 8
Met Arg Val Glu Glu Leu Gly Ile Glu Gly Val Phe Thr Phe Thr Pro
1 5 10 15
Gln Thr Phe Ala Asp Glu Arg Gly Val Phe Gly Thr Ala Tyr Gln Glu
20 25 30
Asp Val Phe Val Ala Ala Leu Gly Arg Pro Leu Phe Pro Val Ala Gln
35 40 45
Val Ser Thr Thr Arg Ser Arg Arg Gly Val Val Arg Gly Val His Phe
50 55 60
Thr Thr Met Pro Gly Ser Met Ala Lys Tyr Val Tyr Cys Ala Arg Gly

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65                  70                  75                  80
Arg Ala Met Asp Phe Ala Val Asp Ile Arg Pro Gly Ser Pro Thr Phe
                85                  90                  95
Gly Arg Ala Glu Pro Val Glu Leu Ser Ala Glu Ser Met Val Gly Leu
            100                 105                 110
Tyr Leu Pro Val Gly Met Gly His Leu Phe Val Ser Leu Glu Asp Asp
        115                 120                 125
Thr Thr Leu Val Tyr Leu Met Ser Ala Gly Tyr Val Pro Asp Lys Glu
    130                 135                 140
Arg Ala Val His Pro Leu Asp Pro Glu Leu Ala Leu Pro Ile Pro Ala
145                 150                 155                 160
Asp Leu Asp Leu Val Met Ser Glu Arg Asp Arg Val Ala Pro Thr Leu
                165                 170                 175
Arg Glu Ala Arg Asp Gln Gly Ile Leu Pro Asp Tyr Ala Ala Cys Arg
            180                 185                 190
Ala Ala Ala His Arg Val Val Arg Thr
        195                 200

<210> 9
<211> 328
<212> PRT
<213> Micromonospora megalomicea

<400> 9
Met Val Val Leu Gly Ala Ser Gly Phe Leu Gly Ser Ala Val Thr His
 1               5                  10                  15
Ala Leu Ala Asp Leu Pro Val Arg Val Arg Leu Val Ala Arg Arg Glu
            20                  25                  30
Val Val Val Pro Ser Gly Ala Val Ala Asp Tyr Glu Thr His Arg Val
        35                  40                  45
Asp Leu Thr Glu Pro Gly Ala Leu Ala Glu Val Val Ala Asp Ala Arg
    50                  55                  60
Ala Val Phe Pro Phe Ala Ala Gln Ile Arg Gly Thr Ser Gly Trp Arg
65                  70                  75                  80
Ile Ser Glu Asp Asp Val Val Ala Glu Arg Thr Asn Val Gly Leu Val
                85                  90                  95
Arg Asp Leu Ile Ala Val Leu Ser Arg Ser Pro His Ala Pro Val Val
            100                 105                 110
Val Phe Pro Gly Ser Asn Thr Gln Val Gly Arg Val Thr Ala Gly Arg
        115                 120                 125
Val Ile Asp Gly Ser Glu Gln Asp His Pro Glu Gly Val Tyr Asp Arg
    130                 135                 140
Gln Lys His Thr Gly Glu Gln Leu Leu Lys Glu Ala Thr Ala Ala Gly
145                 150                 155                 160
Ala Ile Arg Ala Thr Ser Leu Arg Leu Pro Pro Val Phe Gly Val Pro
                165                 170                 175
Ala Ala Gly Thr Ala Asp Asp Arg Gly Val Val Ser Thr Met Ile Arg
            180                 185                 190
Arg Ala Leu Thr Gly Gln Pro Leu Thr Met Trp His Asp Gly Thr Val
        195                 200                 205
Arg Arg Glu Leu Leu Tyr Val Thr Asp Ala Ala Arg Ala Phe Val Thr
    210                 215                 220
Ala Leu Asp His Ala Asp Ala Leu Ala Gly Arg His Phe Leu Leu Gly
225                 230                 235                 240
Thr Gly Arg Ser Trp Pro Leu Gly Glu Val Phe Gln Ala Val Ser Arg
                245                 250                 255
Ser Val Ala Arg His Thr Gly Glu Asp Pro Val Pro Val Val Ser Val
            260                 265                 270
Pro Pro Pro Ala His Met Asp Pro Ser Asp Leu Arg Ser Val Glu Val
        275                 280                 285
Asp Pro Ala Arg Phe Thr Ala Val Thr Gly Trp Arg Ala Thr Val Thr
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290 295 300
Met Ala Glu Ala Val Asp Arg Thr Val Ala Ala Leu Ala Pro Arg Arg
305 310 315 320
Ala Ala Ala Pro Ser Glu Pro Ser
325

<210> 10
<211> 330
<212> PRT
<213> Micromonospora megalomicea

<400> 10
Met Gly Thr Thr Gly Ala Gly Ser Ala Arg Val Arg Val Gly Arg Ser
1 5 10 15
Ala Leu His Thr Ser Arg Leu Trp Leu Gly Thr Val Asn Phe Ser Gly
20 25 30
Arg Val Thr Asp Asp Asp Ala Leu Arg Leu Met Asp His Ala Leu Glu
35 40 45
Arg Gly Val Asn Cys Ile Asp Thr Ala Asp Ile Tyr Gly Trp Arg Leu
50 55 60
Tyr Lys Gly His Thr Glu Glu Leu Val Gly Arg Trp Phe Ala Gln Gly
65 70 75 80
Gly Gly Arg Arg Glu Glu Thr Val Leu Ala Thr Lys Val Gly Ser Glu
85 90 95
Met Ser Glu Arg Val Asn Asp Gly Gly Leu Ser Ala Arg His Ile Val
100 105 110
Ala Ala Cys Glu Asn Ser Leu Arg Arg Leu Gly Val Asp His Ile Asp
115 120 125
Ile Tyr Gln Thr His His Ile Asp Arg Ala Ala Pro Trp Asp Glu Val
130 135 140
Trp Gln Ala Ala Glu His Leu Val Gly Ser Gly Lys Val Gly Tyr Val
145 150 155 160
Gly Ser Ser Asn Leu Ala Gly Trp His Ile Ala Ala Ala Gln Glu Ser
165 170 175
Ala Ala Arg Arg Asn Leu Leu Gly Met Ile Ser His Gln Cys Leu Tyr
180 185 190
Asn Leu Ala Val Arg His Pro Glu Leu Asp Val Leu Pro Ala Ala Gln
195 200 205
Ala Tyr Gly Val Gly Val Phe Ala Trp Ser Pro Leu His Gly Gly Leu
210 215 220
Leu Ser Gly Val Leu Glu Lys Leu Ala Ala Gly Thr Ala Val Lys Ser
225 230 235 240
Ala Gln Gly Arg Ala Gln Val Leu Leu Pro Ala Val Arg Pro Leu Val
245 250 255
Glu Ala Tyr Glu Asp Tyr Cys Arg Arg Leu Gly Ala Asp Pro Ala Glu
260 265 270
Val Gly Leu Ala Trp Val Leu Ser Arg Pro Gly Ile Leu Gly Ala Val
275 280 285
Ile Gly Pro Arg Thr Pro Glu Gln Leu Asp Ser Ala Leu Arg Ala Ala
290 295 300
Glu Leu Thr Leu Gly Glu Glu Glu Leu Arg Glu Leu Glu Ala Ile Phe
305 310 315 320
Pro Ala Pro Ala Val Asp Gly Pro Val Pro
325 330

<210> 11
<211> 417
<212> PRT
<213> Micromonospora megalomicea

<400> 11

Met Arg Val Leu Leu Thr Ser Phe Ala His Arg Thr His Phe Gln Gly
1 5 10 15
Leu Val Pro Leu Ala Trp Ala Leu His Thr Ala Gly His Asp Val Arg
20 25 30
Val Ala Ser Gln Pro Glu Leu Thr Asp Val Val Val Gly Ala Gly Leu
35 40 45
Thr Ser Val Pro Leu Gly Ser Asp His Arg Leu Phe Asp Ile Ser Pro
50 55 60
Glu Ala Ala Ala Gln Val His Arg Tyr Thr Thr Asp Leu Asp Phe Ala
65 70 75 80
Arg Arg Gly Pro Glu Leu Arg Ser Trp Glu Phe Leu His Gly Ile Glu
85 90 95
Glu Ala Thr Ser Arg Phe Val Phe Pro Val Val Asn Asn Asp Ser Phe
100 105 110
Val Asp Glu Leu Val Glu Phe Ala Met Asp Trp Arg Pro Asp Leu Val
115 120 125
Leu Trp Glu Pro Phe Thr Phe Ala Gly Ala Val Ala Ala Lys Ala Cys
130 135 140
Gly Ala Ala His Ala Arg Leu Leu Trp Gly Ser Asp Leu Thr Gly Tyr
145 150 155 160
Phe Arg Ser Arg Ser Gln Asp Leu Arg Gly Gln Arg Pro Ala Asp Asp
165 170 175
Arg Pro Asp Pro Leu Gly Gly Trp Leu Thr Glu Val Ala Gly Arg Phe
180 185 190
Gly Leu Asp Tyr Ser Glu Asp Leu Ala Val Gly Gln Trp Ser Val Asp
195 200 205
Gln Leu Pro Glu Ser Phe Arg Leu Glu Thr Gly Leu Glu Ser Val His
210 215 220
Thr Arg Thr Leu Pro Tyr Asn Gly Ser Ser Val Val Pro Gln Trp Leu
225 230 235 240
Arg Thr Ser Asp Gly Val Arg Arg Val Cys Phe Thr Gly Gly Tyr Ser
245 250 255
Ala Leu Gly Ile Thr Ser Asn Pro Gln Glu Phe Leu Arg Thr Leu Ala
260 265 270
Thr Leu Ala Arg Phe Asp Gly Glu Ile Val Val Thr Arg Ser Gly Leu
275 280 285
Asp Pro Ala Ser Val Pro Asp Asn Val Arg Leu Val Asp Phe Val Pro
290 295 300
Met Asn Ile Leu Leu Pro Gly Cys Ala Ala Val Ile His His Gly Gly
305 310 315 320
Ala Gly Ser Trp Ala Thr Ala Leu His His Gly Val Pro Gln Ile Ser
325 330 335
Val Ala His Glu Trp Asp Cys Val Leu Arg Gly Gln Arg Thr Ala Glu
340 345 350
Leu Gly Ala Gly Val Phe Leu Arg Pro Asp Glu Val Asp Ala Asp Thr
355 360 365
Leu Trp Gln Ala Leu Ala Thr Val Val Glu Asp Arg Ser His Ala Glu
370 375 380
Asn Ala Glu Lys Leu Arg Gln Glu Ala Leu Ala Ala Pro Thr Pro Ala
385 390 395 400
Glu Val Val Pro Val Leu Glu Ala Leu Ala His Gln His Arg Ala Asp
405 410 415

Arg

<210> 12
<211> 313
<212> PRT
<213> Micromonospora megalomicea

<400> 12

Met Thr Arg His Val Thr Leu Leu Gly Val Ser Gly Phe Val Gly Ser
1 5 10 15
Ala Leu Leu Arg Glu Phe Thr Thr His Pro Leu Arg Leu Arg Ala Val
20 25 30
Ala Arg Thr Gly Ser Arg Asp Gln Pro Pro Gly Ser Ala Gly Ile Glu
35 40 45
His Leu Arg Val Asp Leu Leu Glu Pro Gly Arg Val Ala Gln Val Val
50 55 60
Ala Asp Thr Asp Val Val Val His Leu Val Ala Tyr Ala Ala Gly Gly
65 70 75 80
Ser Thr Trp Arg Ser Ala Ala Thr Val Pro Glu Ala Glu Arg Val Asn
85 90 95
Ala Gly Ile Met Arg Asp Leu Val Ala Ala Leu Arg Ala Arg Pro Gly
100 105 110
Pro Ala Pro Val Leu Leu Phe Ala Ser Thr Thr Gln Ala Ala Asn Pro
115 120 125
Ala Ala Pro Ser Arg Tyr Ala Gln His Lys Ile Glu Ala Glu Arg Ile
130 135 140
Leu Arg Gln Ala Thr Glu Asp Gly Val Val Asp Gly Val Ile Leu Arg
145 150 155 160
Leu Pro Ala Ile Tyr Gly His Ser Gly Pro Ser Gly Gln Thr Gly Arg
165 170 175
Gly Val Val Thr Ala Met Ile Arg Arg Ala Leu Ala Gly Glu Pro Ile
180 185 190
Thr Met Trp His Glu Gly Ser Val Arg Arg Asn Leu Leu His Val Glu
195 200 205
Asp Val Ala Thr Ala Phe Thr Ala Ala Leu His Asn His Glu Ala Leu
210 215 220
Val Gly Asp Val Trp Thr Pro Ser Ala Asp Glu Ala Arg Pro Leu Gly
225 230 235 240
Glu Ile Phe Glu Thr Val Ala Ala Ser Val Ala Arg Gln Thr Gly Asn
245 250 255
Pro Ala Val Pro Val Val Ser Val Pro Pro Pro Glu Asn Ala Glu Ala
260 265 270
Asn Asp Phe Arg Ser Asp Asp Phe Asp Ser Thr Glu Phe Arg Thr Leu
275 280 285
Thr Gly Trp His Pro Arg Val Pro Leu Ala Glu Gly Ile Asp Arg Thr
290 295 300
Val Ala Ala Leu Ile Ser Thr Lys Glu
305 310

<210> 13
<211> 3546
<212> PRT
<213> Micromonospora megalomicea

<400> 13
Met Val Asp Val Pro Asp Leu Leu Gly Thr Arg Thr Pro His Pro Gly
1 5 10 15
Pro Leu Pro Phe Pro Trp Pro Leu Cys Gly His Asn Glu Pro Glu Leu
20 25 30
Arg Ala Arg Ala Arg Gln Leu His Ala Tyr Leu Glu Gly Ile Ser Glu
35 40 45
Asp Asp Val Val Ala Val Gly Ala Ala Leu Ala Arg Glu Thr Arg Ala
50 55 60
Gln Asp Gly Pro His Arg Ala Val Val Val Ala Ser Ser Val Thr Glu
65 70 75 80
Leu Thr Ala Ala Leu Ala Ala Leu Ala Gln Gly Arg Pro His Pro Ser
85 90 95
Val Val Arg Gly Val Ala Arg Pro Thr Ala Pro Val Val Phe Val Leu
100 105 110

```
Pro Gly Gln Gly Ala Gln Trp Pro Gly Met Ala Thr Arg Leu Leu Ala
        115                 120                 125
Glu Ser Pro Val Phe Ala Ala Ala Met Arg Ala Cys Glu Arg Ala Phe
    130                 135                 140
Asp Glu Val Thr Asp Trp Ser Leu Thr Glu Val Leu Asp Ser Pro Glu
145                 150                 155                 160
His Leu Arg Arg Val Glu Val Val Gln Pro Ala Leu Phe Ala Val Gln
                165                 170                 175
Thr Ser Leu Ala Ala Leu Trp Arg Ser Phe Gly Val Arg Pro Asp Ala
            180                 185                 190
Val Leu Gly His Ser Ile Gly Glu Leu Ala Ala Ala Glu Val Cys Gly
        195                 200                 205
Ala Val Asp Val Glu Ala Ala Ala Arg Ala Ala Ala Leu Trp Ser Arg
    210                 215                 220
Glu Met Val Pro Leu Val Gly Arg Gly Asp Met Ala Ala Val Ala Leu
225                 230                 235                 240
Ser Pro Ala Glu Leu Ala Ala Arg Val Glu Arg Trp Asp Asp Asp Val
                245                 250                 255
Val Pro Ala Gly Val Asn Gly Pro Arg Ser Val Leu Leu Thr Gly Ala
            260                 265                 270
Pro Glu Pro Ile Ala Arg Arg Val Ala Glu Leu Ala Ala Gln Gly Val
        275                 280                 285
Arg Ala Gln Val Val Asn Val Ser Met Ala Ala His Ser Ala Gln Val
    290                 295                 300
Asp Ala Val Ala Glu Gly Met Arg Ser Ala Leu Thr Trp Phe Ala Pro
305                 310                 315                 320
Gly Asp Ser Asp Val Pro Tyr Tyr Ala Gly Leu Thr Gly Gly Arg Leu
                325                 330                 335
Asp Thr Arg Glu Leu Gly Ala Asp His Trp Pro Arg Ser Phe Arg Leu
            340                 345                 350
Pro Val Arg Phe Asp Glu Ala Thr Arg Ala Val Leu Glu Leu Gln Pro
        355                 360                 365
Gly Thr Phe Ile Glu Ser Ser Pro His Pro Val Leu Ala Ala Ser Leu
    370                 375                 380
Gln Gln Thr Leu Asp Glu Val Gly Ser Pro Ala Ala Ile Val Pro Thr
385                 390                 395                 400
Leu Gln Arg Asp Gln Gly Gly Leu Arg Arg Phe Leu Leu Ala Val Ala
                405                 410                 415
Gln Ala Tyr Thr Gly Gly Val Thr Val Asp Trp Thr Ala Ala Tyr Pro
            420                 425                 430
Gly Val Thr Pro Gly His Leu Pro Ser Ala Val Ala Val Glu Thr Asp
        435                 440                 445
Glu Gly Pro Ser Thr Glu Phe Asp Trp Ala Ala Pro Asp His Val Leu
    450                 455                 460
Arg Ala Arg Leu Leu Glu Ile Val Gly Ala Glu Thr Ala Ala Leu Ala
465                 470                 475                 480
Gly Arg Glu Val Asp Ala Arg Ala Thr Phe Arg Glu Leu Gly Leu Asp
                485                 490                 495
Ser Val Leu Ala Val Gln Leu Arg Thr Arg Leu Ala Thr Ala Thr Gly
            500                 505                 510
Arg Asp Leu His Ile Ala Met Leu Tyr Asp His Pro Thr Pro His Ala
        515                 520                 525
Leu Thr Glu Ala Leu Leu Arg Gly Pro Gln Glu Glu Pro Gly Arg Gly
    530                 535                 540
Glu Glu Thr Ala His Pro Thr Glu Ala Glu Pro Asp Glu Pro Val Ala
545                 550                 555                 560
Val Val Ala Met Ala Cys Arg Leu Pro Gly Gly Val Thr Ser Pro Glu
                565                 570                 575
Glu Phe Trp Glu Leu Leu Ala Glu Gly Arg Asp Ala Val Gly Gly Leu
            580                 585                 590
Pro Thr Asp Arg Gly Trp Asp Leu Asp Ser Leu Phe His Pro Asp Pro
```

595 600 605
Thr Arg Ser Gly Thr Ala His Gln Arg Ala Gly Gly Phe Leu Thr Gly
610 615 620
Ala Thr Ser Phe Asp Ala Ala Phe Phe Gly Leu Ser Pro Arg Glu Ala
625 630 635 640
Leu Ala Val Glu Pro Gln Gln Arg Ile Thr Leu Glu Leu Ser Trp Glu
645 650 655
Val Leu Glu Arg Ala Gly Ile Pro Pro Thr Ser Leu Arg Thr Ser Arg
660 665 670
Thr Gly Val Phe Val Gly Leu Ile Pro Gln Glu Tyr Gly Pro Arg Leu
675 680 685
Ala Glu Gly Gly Glu Gly Val Glu Gly Tyr Leu Met Thr Gly Thr Thr
690 695 700
Thr Ser Val Ala Ser Gly Arg Val Ala Tyr Thr Leu Gly Leu Glu Gly
705 710 715 720
Pro Ala Ile Ser Val Asp Thr Ala Cys Ser Ser Ser Leu Val Ala Val
725 730 735
His Leu Ala Cys Gln Ser Leu Arg Arg Gly Glu Ser Thr Met Ala Leu
740 745 750
Ala Gly Gly Val Thr Val Met Pro Thr Pro Gly Met Leu Val Asp Phe
755 760 765
Ser Arg Met Asn Ser Leu Ala Pro Asp Gly Arg Ser Lys Ala Phe Ser
770 775 780
Ala Ala Ala Asp Gly Phe Gly Met Ala Glu Gly Ala Gly Met Leu Leu
785 790 795 800
Leu Glu Arg Leu Ser Asp Ala Arg Arg His Gly His Pro Val Leu Ala
805 810 815
Val Ile Arg Gly Thr Ala Val Asn Ser Asp Gly Ala Ser Asn Gly Leu
820 825 830
Ser Ala Pro Asn Gly Arg Ala Gln Val Arg Val Ile Arg Gln Ala Leu
835 840 845
Ala Glu Ser Gly Leu Thr Pro His Thr Val Asp Val Val Glu Thr His
850 855 860
Gly Thr Gly Thr Arg Leu Gly Asp Pro Ile Glu Ala Arg Ala Leu Ser
865 870 875 880
Asp Ala Tyr Gly Gly Asp Arg Glu His Pro Leu Arg Ile Gly Ser Val
885 890 895
Lys Ser Asn Ile Gly His Thr Gln Ala Ala Ala Gly Val Ala Gly Leu
900 905 910
Ile Lys Leu Val Leu Ala Met Gln Ala Gly Val Leu Pro Arg Thr Leu
915 920 925
His Ala Asp Glu Pro Ser Pro Glu Ile Asp Trp Ser Ser Gly Ala Ile
930 935 940
Ser Leu Leu Gln Glu Pro Ala Ala Trp Pro Ala Gly Glu Arg Pro Arg
945 950 955 960
Arg Ala Gly Val Ser Ser Phe Gly Ile Ser Gly Thr Asn Ala His Ala
965 970 975
Ile Ile Glu Glu Ala Pro Pro Thr Gly Asp Asp Thr Arg Pro Asp Arg
980 985 990
Met Gly Pro Val Val Pro Trp Val Leu Ser Ala Ser Thr Gly Glu Ala
995 1000 1005
Leu Arg Ala Arg Ala Ala Arg Leu Ala Gly His Leu Arg Glu His Pro
1010 1015 1020
Asp Gln Asp Leu Asp Asp Val Ala Tyr Ser Leu Ala Thr Gly Arg Ala
1025 1030 1035 1040
Ala Leu Ala Tyr Arg Ser Gly Phe Val Pro Ala Asp Ala Ser Thr Ala
1045 1050 1055
Leu Arg Ile Leu Asp Glu Leu Ala Ala Gly Gly Ser Gly Asp Ala Val
1060 1065 1070
Thr Gly Thr Ala Arg Ala Pro Gln Arg Val Val Phe Val Phe Pro Gly
1075 1080 1085

Gln Gly Trp Gln Trp Ala Gly Met Ala Val Asp Leu Leu Asp Gly Asp
1090 1095 1100
Pro Val Phe Ala Ser Val Leu Arg Glu Cys Ala Asp Ala Leu Glu Pro
1105 1110 1115 1120
Tyr Leu Asp Phe Glu Ile Val Pro Phe Leu Arg Ala Glu Ala Gln Arg
1125 1130 1135
Arg Thr Pro Asp His Thr Leu Ser Thr Asp Arg Val Asp Val Val Gln
1140 1145 1150
Pro Val Leu Phe Ala Val Met Val Ser Leu Ala Ala Arg Trp Arg Ala
1155 1160 1165
Tyr Gly Val Glu Pro Ala Ala Val Ile Gly His Ser Gln Gly Glu Ile
1170 1175 1180
Ala Ala Ala Cys Val Ala Gly Ala Leu Ser Leu Asp Asp Ala Ala Arg
1185 1190 1195 1200
Ala Val Ala Leu Arg Ser Arg Val Ile Ala Thr Met Pro Gly Asn Gly
1205 1210 1215
Ala Met Ala Ser Ile Ala Ala Ser Val Asp Glu Val Ala Ala Arg Ile
1220 1225 1230
Asp Gly Arg Val Glu Ile Ala Ala Val Asn Gly Pro Arg Ala Val Val
1235 1240 1245
Val Ser Gly Asp Arg Asp Asp Leu Asp Arg Leu Val Ala Ser Cys Thr
1250 1255 1260
Val Glu Gly Val Arg Ala Lys Arg Leu Pro Val Asp Tyr Ala Ser His
1265 1270 1275 1280
Ser Ser His Val Glu Ala Val Arg Asp Ala Leu His Ala Glu Leu Gly
1285 1290 1295
Glu Phe Arg Pro Leu Pro Gly Phe Val Pro Phe Tyr Ser Thr Val Thr
1300 1305 1310
Gly Arg Trp Val Glu Pro Ala Glu Leu Asp Ala Gly Tyr Trp Phe Arg
1315 1320 1325
Asn Leu Arg His Arg Val Arg Phe Ala Asp Ala Val Arg Ser Leu Ala
1330 1335 1340
Asp Gln Gly Tyr Thr Thr Phe Leu Glu Val Ser Ala His Pro Val Leu
1345 1350 1355 1360
Thr Thr Ala Ile Glu Glu Ile Gly Glu Asp Arg Gly Gly Asp Leu Val
1365 1370 1375
Ala Val His Ser Leu Arg Arg Gly Ala Gly Gly Pro Val Asp Phe Gly
1380 1385 1390
Ser Ala Leu Ala Arg Ala Phe Val Ala Gly Val Ala Val Asp Trp Glu
1395 1400 1405
Ser Ala Tyr Gln Gly Ala Gly Ala Arg Arg Val Pro Leu Pro Thr Tyr
1410 1415 1420
Pro Phe Gln Arg Glu Arg Phe Trp Leu Glu Pro Asn Pro Ala Arg Arg
1425 1430 1435 1440
Val Ala Asp Ser Asp Asp Val Ser Ser Leu Arg Tyr Arg Ile Glu Trp
1445 1450 1455
His Pro Thr Asp Pro Gly Glu Pro Gly Arg Leu Asp Gly Thr Trp Leu
1460 1465 1470
Leu Ala Thr Tyr Pro Gly Arg Ala Asp Asp Arg Val Glu Ala Ala Arg
1475 1480 1485
Gln Ala Leu Glu Ser Ala Gly Ala Arg Val Glu Asp Leu Val Val Glu
1490 1495 1500
Pro Arg Thr Gly Arg Val Asp Leu Val Arg Arg Leu Asp Ala Val Gly
1505 1510 1515 1520
Pro Val Ala Gly Val Leu Cys Leu Phe Ala Val Ala Glu Pro Ala Ala
1525 1530 1535
Glu His Ser Pro Leu Ala Val Thr Ser Leu Ser Asp Thr Leu Asp Leu
1540 1545 1550
Thr Gln Ala Val Ala Gly Ser Gly Arg Glu Cys Pro Ile Trp Val Val
1555 1560 1565
Thr Glu Asn Ala Val Ala Val Gly Pro Phe Glu Arg Leu Arg Asp Pro

1570 1575 1580
Ala His Gly Ala Leu Trp Ala Leu Gly Arg Val Val Ala Leu Glu Asn
1585 1590 1595 1600
Pro Ala Val Trp Gly Gly Leu Val Asp Val Pro Ser Gly Ser Val Ala
1605 1610 1615
Glu Leu Ser Arg His Leu Gly Thr Thr Leu Ser Gly Ala Gly Glu Asp
1620 1625 1630
Gln Val Ala Leu Arg Pro Asp Gly Thr Tyr Ala Arg Arg Trp Cys Arg
1635 1640 1645
Ala Gly Ala Gly Gly Thr Gly Arg Trp Gln Pro Arg Gly Thr Val Leu
1650 1655 1660
Val Thr Gly Gly Thr Gly Gly Val Gly Arg His Val Ala Arg Trp Leu
1665 1670 1675 1680
Ala Arg Gln Gly Thr Pro Cys Leu Val Leu Ala Ser Arg Arg Gly Pro
1685 1690 1695
Asp Ala Asp Gly Val Glu Glu Leu Leu Thr Glu Leu Ala Asp Leu Gly
1700 1705 1710
Thr Arg Ala Thr Val Thr Ala Cys Asp Val Thr Asp Arg Glu Gln Leu
1715 1720 1725
Arg Ala Leu Leu Ala Thr Val Asp Asp Glu His Pro Leu Ser Ala Val
1730 1735 1740
Phe His Val Ala Ala Thr Leu Asp Asp Gly Thr Val Glu Thr Leu Thr
1745 1750 1755 1760
Gly Asp Arg Ile Glu Arg Ala Asn Arg Ala Lys Val Leu Gly Ala Arg
1765 1770 1775
Asn Leu His Glu Leu Thr Arg Asp Ala Asp Leu Asp Ala Phe Val Leu
1780 1785 1790
Phe Ser Ser Ser Thr Ala Ala Phe Gly Ala Pro Gly Leu Gly Gly Tyr
1795 1800 1805
Val Pro Gly Asn Ala Tyr Leu Asp Gly Leu Ala Gln Gln Arg Arg Ser
1810 1815 1820
Glu Gly Leu Pro Ala Thr Ser Val Ala Trp Gly Thr Trp Ala Gly Ser
1825 1830 1835 1840
Gly Met Ala Glu Gly Pro Val Ala Asp Arg Phe Arg Arg His Gly Val
1845 1850 1855
Met Glu Met His Pro Asp Gln Ala Val Glu Gly Leu Arg Val Ala Leu
1860 1865 1870
Val Gln Gly Glu Val Ala Pro Ile Val Val Asp Ile Arg Trp Asp Arg
1875 1880 1885
Phe Leu Leu Ala Tyr Thr Ala Gln Arg Pro Thr Arg Leu Phe Asp Thr
1890 1895 1900
Leu Asp Glu Ala Arg Arg Ala Ala Pro Gly Pro Asp Ala Gly Pro Gly
1905 1910 1915 1920
Val Ala Ala Leu Ala Gly Leu Pro Val Gly Glu Arg Glu Lys Ala Val
1925 1930 1935
Leu Asp Leu Val Arg Thr His Ala Ala Ala Val Leu Gly His Ala Ser
1940 1945 1950
Ala Glu Gln Val Pro Val Asp Arg Ala Phe Ala Glu Leu Gly Val Asp
1955 1960 1965
Ser Leu Ser Ala Leu Glu Leu Arg Asn Arg Leu Thr Thr Ala Thr Gly
1970 1975 1980
Val Arg Leu Ala Thr Thr Thr Val Phe Asp His Pro Asp Val Arg Thr
1985 1990 1995 2000
Leu Ala Gly His Leu Ala Ala Glu Leu Gly Gly Gly Ser Gly Arg Glu
2005 2010 2015
Arg Pro Gly Gly Glu Ala Pro Thr Val Ala Pro Thr Asp Glu Pro Ile
2020 2025 2030
Ala Ile Val Gly Met Ala Cys Arg Leu Pro Gly Gly Val Asp Ser Pro
2035 2040 2045
Glu Gln Leu Trp Glu Leu Ile Val Ser Gly Arg Asp Thr Ala Ser Ala
2050 2055 2060

Ala Pro Gly Asp Arg Ser Trp Asp Pro Ala Glu Leu Met Val Ser Asp
2065 2070 2075 2080
Thr Thr Gly Thr Arg Thr Ala Phe Gly Asn Phe Met Pro Gly Ala Gly
2085 2090 2095
Glu Phe Asp Ala Ala Phe Phe Gly Ile Ser Pro Arg Glu Ala Leu Ala
2100 2105 2110
Met Asp Pro Gln Gln Arg His Ala Leu Glu Thr Thr Trp Glu Ala Leu
2115 2120 2125
Glu Asn Ala Gly Ile Arg Pro Glu Ser Leu Arg Gly Thr Asp Thr Gly
2130 2135 2140
Val Phe Val Gly Met Ser His Gln Gly Tyr Ala Thr Gly Arg Pro Lys
2145 2150 2155 2160
Pro Glu Asp Glu Val Asp Gly Tyr Leu Leu Thr Gly Asn Thr Ala Ser
2165 2170 2175
Val Ala Ser Gly Arg Ile Ala Tyr Val Leu Gly Leu Glu Gly Pro Ala
2180 2185 2190
Ile Thr Val Asp Thr Ala Cys Ser Ser Ser Leu Val Ala Leu His Val
2195 2200 2205
Ala Ala Gly Ser Leu Arg Ser Gly Asp Cys Gly Leu Ala Val Ala Gly
2210 2215 2220
Gly Val Ser Val Met Ala Gly Pro Glu Val Phe Arg Glu Phe Ser Arg
2225 2230 2235 2240
Gln Gly Ala Leu Ala Pro Asp Gly Arg Cys Lys Pro Phe Ser Asp Glu
2245 2250 2255
Ala Asp Gly Phe Gly Leu Gly Glu Gly Ser Ala Phe Val Val Leu Gln
2260 2265 2270
Arg Leu Ser Val Ala Val Arg Glu Gly Arg Arg Val Leu Gly Val Val
2275 2280 2285
Val Gly Ser Ala Val Asn Gln Asp Gly Ala Ser Asn Gly Leu Ala Ala
2290 2295 2300
Pro Ser Gly Val Ala Gln Gln Arg Val Ile Arg Arg Ala Trp Gly Arg
2305 2310 2315 2320
Ala Gly Val Ser Gly Gly Asp Val Gly Val Val Glu Ala His Gly Thr
2325 2330 2335
Gly Thr Arg Leu Gly Asp Pro Val Glu Leu Gly Ala Leu Leu Gly Thr
2340 2345 2350
Tyr Gly Val Gly Arg Gly Gly Val Gly Pro Val Val Val Gly Ser Val
2355 2360 2365
Lys Ala Asn Val Gly His Val Gln Ala Ala Ala Gly Val Val Gly Val
2370 2375 2380
Ile Lys Val Val Leu Gly Leu Gly Arg Gly Leu Val Gly Pro Met Val
2385 2390 2395 2400
Cys Arg Gly Gly Leu Ser Gly Leu Val Asp Trp Ser Ser Gly Gly Leu
2405 2410 2415
Val Val Ala Asp Gly Val Arg Gly Trp Pro Val Gly Val Asp Gly Val
2420 2425 2430
Arg Arg Gly Gly Val Ser Ala Phe Gly Val Ser Gly Thr Asn Ala His
2435 2440 2445
Val Val Val Ala Glu Ala Pro Gly Ser Val Val Gly Ala Glu Arg Pro
2450 2455 2460
Val Glu Gly Ser Ser Arg Gly Leu Val Gly Val Val Gly Gly Val Val
2465 2470 2475 2480
Pro Val Val Leu Ser Ala Lys Thr Glu Thr Ala Leu His Ala Gln Ala
2485 2490 2495
Arg Arg Leu Ala Asp His Leu Glu Thr His Pro Asp Val Pro Met Thr
2500 2505 2510
Asp Val Val Trp Thr Leu Thr Gln Ala Arg Gln Arg Phe Asp Arg Arg
2515 2520 2525
Ala Val Leu Leu Ala Ala Asp Arg Thr Gln Ala Val Glu Arg Leu Arg
2530 2535 2540
Gly Leu Ala Gly Gly Glu Pro Gly Thr Gly Val Val Ser Gly Val Ala

2545 2550 2555 2560
Ser Gly Gly Gly Val Val Phe Val Phe Pro Gly Gln Gly Gly Gln Trp
2565 2570 2575
Val Gly Met Ala Arg Gly Leu Leu Ser Val Pro Val Phe Val Glu Ser
2580 2585 2590
Val Val Glu Cys Asp Ala Val Val Ser Ser Val Val Gly Phe Ser Val
2595 2600 2605
Leu Gly Val Leu Glu Gly Arg Ser Gly Ala Pro Ser Leu Asp Arg Val
2610 2615 2620
Asp Val Val Gln Pro Val Leu Phe Val Val Met Val Ser Leu Ala Arg
2625 2630 2635 2640
Leu Trp Arg Trp Cys Gly Val Val Pro Ala Ala Val Val Gly His Ser
2645 2650 2655
Gln Gly Glu Ile Ala Ala Ala Val Val Ala Gly Val Leu Ser Val Gly
2660 2665 2670
Asp Gly Ala Arg Val Val Ala Leu Arg Ala Arg Ala Leu Arg Ala Leu
2675 2680 2685
Ala Gly His Gly Gly Met Ala Ser Val Arg Arg Gly Arg Asp Asp Val
2690 2695 2700
Gln Lys Leu Leu Asp Ser Gly Pro Trp Thr Gly Lys Leu Glu Ile Ala
2705 2710 2715 2720
Ala Val Asn Gly Pro Asp Ala Val Val Val Ser Gly Asp Pro Arg Ala
2725 2730 2735
Val Thr Glu Leu Val Glu His Cys Asp Gly Ile Gly Val Arg Ala Arg
2740 2745 2750
Thr Ile Pro Val Asp Tyr Ala Ser His Ser Ala Gln Val Glu Ser Leu
2755 2760 2765
Arg Glu Glu Leu Leu Ser Val Leu Ala Gly Ile Glu Gly Arg Pro Ala
2770 2775 2780
Thr Val Pro Phe Tyr Ser Thr Leu Thr Gly Gly Phe Val Asp Gly Thr
2785 2790 2795 2800
Glu Leu Asp Ala Asp Tyr Trp Tyr Arg Asn Leu Arg His Pro Val Arg
2805 2810 2815
Phe His Ala Ala Val Glu Ala Leu Ala Ala Arg Asp Leu Thr Thr Phe
2820 2825 2830
Val Glu Val Ser Pro His Pro Val Leu Ser Met Ala Val Gly Glu Thr
2835 2840 2845
Leu Ala Asp Val Glu Ser Ala Val Thr Val Gly Thr Leu Glu Arg Asp
2850 2855 2860
Thr Asp Asp Val Glu Arg Phe Leu Thr Ser Leu Ala Glu Ala His Val
2865 2870 2875 2880
His Gly Val Pro Val Asp Trp Ala Ala Val Leu Gly Ser Gly Thr Leu
2885 2890 2895
Val Asp Leu Pro Thr Tyr Pro Phe Gln Gly Arg Arg Phe Trp Leu His
2900 2905 2910
Pro Asp Arg Gly Pro Arg Asp Asp Val Ala Asp Trp Phe His Arg Val
2915 2920 2925
Asp Trp Thr Ala Thr Ala Thr Asp Gly Ser Ala Arg Leu Asp Gly Arg
2930 2935 2940
Trp Leu Val Val Val Pro Glu Gly Tyr Thr Asp Asp Gly Trp Val Val
2945 2950 2955 2960
Glu Val Arg Ala Ala Leu Ala Ala Gly Gly Ala Glu Pro Val Val Thr
2965 2970 2975
Thr Val Glu Glu Val Thr Asp Arg Val Gly Asp Ser Asp Ala Val Val
2980 2985 2990
Ser Met Leu Gly Leu Ala Asp Asp Gly Ala Ala Glu Thr Leu Ala Leu
2995 3000 3005
Leu Arg Arg Leu Asp Ala Gln Ala Ser Thr Thr Pro Leu Trp Val Val
3010 3015 3020
Thr Val Gly Ala Val Ala Pro Ala Gly Pro Val Gln Arg Pro Glu Gln
3025 3030 3035 3040

Ala Thr Val Trp Gly Leu Ala Leu Val Ala Ser Leu Glu Arg Gly His
3045 3050 3055
Arg Trp Thr Gly Leu Leu Asp Leu Pro Gln Thr Pro Asp Pro Gln Leu
3060 3065 3070
Arg Pro Arg Leu Val Glu Ala Leu Ala Gly Ala Glu Asp Gln Val Ala
3075 3080 3085
Val Arg Ala Asp Ala Val His Ala Arg Arg Ile Val Pro Thr Pro Val
3090 3095 3100
Thr Gly Ala Gly Pro Tyr Thr Ala Pro Gly Gly Thr Ile Leu Val Thr
3105 3110 3115 3120
Gly Gly Thr Ala Gly Leu Gly Ala Val Thr Ala Arg Trp Leu Ala Glu
3125 3130 3135
Arg Gly Ala Glu His Leu Ala Leu Val Ser Arg Arg Gly Pro Gly Thr
3140 3145 3150
Ala Gly Val Asp Glu Val Val Arg Asp Leu Thr Gly Leu Gly Val Arg
3155 3160 3165
Val Ser Val His Ser Cys Asp Val Gly Asp Arg Glu Ser Val Gly Ala
3170 3175 3180
Leu Val Gln Glu Leu Thr Ala Ala Gly Asp Val Val Arg Gly Val Val
3185 3190 3195 3200
His Ala Ala Gly Leu Pro Gln Gln Val Pro Leu Thr Asp Met Asp Pro
3205 3210 3215
Ala Asp Leu Ala Asp Val Val Ala Val Lys Val Asp Gly Ala Val His
3220 3225 3230
Leu Ala Asp Leu Cys Pro Glu Ala Glu Leu Phe Leu Leu Phe Ser Ser
3235 3240 3245
Gly Ala Gly Val Trp Gly Ser Ala Arg Gln Gly Ala Tyr Ala Ala Gly
3250 3255 3260
Asn Ala Phe Leu Asp Ala Phe Ala Arg His Arg Arg Asp Arg Gly Leu
3265 3270 3275 3280
Pro Ala Thr Ser Val Ala Trp Gly Leu Trp Ala Ala Gly Gly Met Thr
3285 3290 3295
Gly Asp Gln Glu Ala Val Ser Phe Leu Arg Glu Arg Gly Val Arg Pro
3300 3305 3310
Met Ser Val Pro Arg Ala Leu Glu Ala Leu Glu Arg Val Leu Thr Ala
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Gly Glu Thr Ala Val Val Val Ala Asp Val Asp Trp Ala Ala Phe Ala
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Glu Ser Tyr Thr Ser Ala Arg Pro Arg Pro Leu Leu His Arg Leu Val
3345 3350 3355 3360
Thr Pro Ala Ala Ala Val Gly Glu Arg Asp Glu Pro Arg Glu Gln Thr
3365 3370 3375
Leu Arg Asp Arg Leu Ala Ala Leu Pro Arg Ala Glu Arg Ser Ala Glu
3380 3385 3390
Leu Val Arg Leu Val Arg Arg Asp Ala Ala Ala Val Leu Gly Ser Asp
3395 3400 3405
Ala Lys Ala Val Pro Ala Thr Thr Pro Phe Lys Asp Leu Gly Phe Asp
3410 3415 3420
Ser Leu Ala Ala Val Arg Phe Arg Asn Arg Leu Ala Ala His Thr Gly
3425 3430 3435 3440
Leu Arg Leu Pro Ala Thr Leu Val Phe Glu His Pro Asn Ala Ala Ala
3445 3450 3455
Val Ala Asp Leu Leu His Asp Arg Leu Gly Glu Ala Gly Glu Pro Thr
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Pro Val Arg Ser Val Gly Ala Gly Leu Ala Ala Leu Glu Gln Ala Leu
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Pro Asp Ala Ser Asp Thr Glu Arg Val Glu Leu Val Glu Arg Leu Glu
3490 3495 3500
Arg Met Leu Ala Gly Leu Arg Pro Glu Ala Gly Ala Gly Ala Asp Ala
3505 3510 3515 3520
Pro Thr Ala Gly Asp Asp Leu Gly Glu Ala Gly Val Asp Glu Leu Leu

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Ile Ala Val Val Gly Met Ala Cys Arg Leu Pro Gly Gly Val His Leu
        35                  40                  45
Pro Gln His Leu Trp Asp Leu Leu Arg Gln Gly His Glu Thr Val Ser
    50                  55                  60
Thr Phe Pro Thr Gly Arg Gly Trp Asp Leu Ala Gly Leu Phe His Pro
65                  70                  75                  80
Asp Pro Asp His Pro Gly Thr Ser Tyr Val Asp Arg Gly Gly Phe Leu
                85                  90                  95
Asp Asp Val Ala Gly Phe Asp Ala Glu Phe Phe Gly Ile Ser Pro Arg
            100                 105                 110
Glu Ala Thr Ala Met Asp Pro Gln Gln Arg Leu Leu Leu Glu Thr Ser
        115                 120                 125
Trp Glu Leu Val Glu Ser Ala Gly Ile Asp Pro His Ser Leu Arg Gly
    130                 135                 140
Thr Pro Thr Gly Val Phe Leu Gly Val Ala Arg Leu Gly Tyr Gly Glu
145                 150                 155                 160
Asn Gly Thr Glu Ala Gly Asp Ala Glu Gly Tyr Ser Val Thr Gly Val
                165                 170                 175
Ala Pro Ala Val Ala Ser Gly Arg Ile Ser Tyr Ala Leu Gly Leu Glu
            180                 185                 190
Gly Pro Ser Ile Ser Val Asp Thr Ala Cys Ser Ser Ser Leu Val Ala
        195                 200                 205
Leu His Leu Ala Val Glu Ser Leu Arg Leu Gly Glu Ser Ser Leu Ala
    210                 215                 220
Val Val Gly Gly Ala Ala Val Met Ala Thr Pro Gly Val Phe Val Asp
225                 230                 235                 240
Phe Ser Arg Gln Arg Ala Leu Ala Ala Asp Gly Arg Ser Lys Ala Phe
                245                 250                 255
Gly Ala Ala Ala Asp Gly Phe Gly Phe Ser Glu Gly Val Ser Leu Val
            260                 265                 270
Leu Leu Glu Arg Leu Ser Glu Ala Glu Ser Asn Gly His Glu Val Leu
        275                 280                 285
Ala Val Ile Arg Gly Ser Ala Leu Asn Gln Asp Gly Ala Ser Asn Gly
    290                 295                 300
Leu Ala Ala Pro Asn Gly Thr Ala Gln Arg Lys Val Ile Arg Gln Ala
305                 310                 315                 320
Leu Arg Asn Cys Gly Leu Thr Pro Ala Asp Val Asp Ala Val Glu Ala
                325                 330                 335
His Gly Thr Gly Thr Thr Leu Gly Asp Pro Ile Glu Ala Asn Ala Leu
            340                 345                 350
Leu Asp Thr Tyr Gly Arg Asp Arg Asp Pro Asp His Pro Leu Trp Leu
        355                 360                 365
Gly Ser Val Lys Ser Asn Ile Gly His Thr Gln Ala Ala Ala Gly Val
    370                 375                 380
Thr Gly Leu Leu Lys Met Val Leu Ala Leu Arg His Glu Glu Leu Pro
385                 390                 395                 400
Ala Thr Leu His Val Asp Glu Pro Thr Pro His Val Asp Trp Ser Ser
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Gly Ala Val Arg Leu Ala Thr Arg Gly Arg Pro Trp Arg Arg Gly Asp
420 425 430
Arg Pro Arg Arg Ala Gly Val Ser Ala Phe Gly Ile Ser Gly Thr Asn
435 440 445
Ala His Val Ile Val Glu Glu Ala Pro Glu Arg Thr Thr Glu Arg Thr
450 455 460
Val Gly Gly Asp Val Gly Pro Val Pro Leu Val Val Ser Ala Arg Ser
465 470 475 480
Ala Ala Ala Leu Arg Ala Gln Ala Ala Gln Val Ala Glu Leu Val Glu
485 490 495
Gly Ser Asp Val Gly Leu Ala Glu Val Gly Arg Ser Leu Ala Val Thr
500 505 510
Arg Ala Arg His Glu His Arg Ala Ala Val Val Ala Ser Thr Arg Ala
515 520 525
Glu Ala Val Arg Gly Leu Arg Glu Val Ala Ala Val Glu Pro Arg Gly
530 535 540
Glu Asp Thr Val Thr Gly Val Ala Glu Thr Ser Gly Arg Thr Val Val
545 550 555 560
Phe Leu Phe Pro Gly Gln Gly Ser Gln Trp Val Gly Met Gly Ala Glu
565 570 575
Leu Leu Asp Ser Ala Pro Ala Phe Ala Asp Thr Ile Arg Ala Cys Asp
580 585 590
Glu Ala Met Ala Pro Leu Gln Asp Trp Ser Val Ser Asp Val Leu Arg
595 600 605
Gln Glu Pro Gly Ala Pro Gly Leu Asp Arg Val Asp Val Val Gln Pro
610 615 620
Val Leu Phe Ala Val Met Val Ser Leu Ala Arg Leu Trp Gln Ser Tyr
625 630 635 640
Gly Val Thr Pro Ala Ala Val Val Gly His Ser Gln Gly Glu Ile Ala
645 650 655
Ala Ala His Val Ala Gly Ala Leu Ser Leu Ala Asp Ala Ala Arg Leu
660 665 670
Val Val Gly Arg Ser Arg Leu Leu Arg Ser Leu Ser Gly Gly Gly Gly
675 680 685
Met Ser Ala Val Ala Leu Gly Glu Ala Glu Val Arg Arg Arg Leu Arg
690 695 700
Ser Trp Glu Asp Arg Ile Ser Val Ala Ala Val Asn Gly Pro Arg Ser
705 710 715 720
Val Val Val Ala Gly Glu Pro Glu Ala Leu Arg Glu Trp Gly Arg Glu
725 730 735
Arg Glu Ala Glu Gly Val Arg Val Arg Glu Ile Asp Val Asp Tyr Ala
740 745 750
Ser His Ser Pro Gln Ile Asp Arg Val Arg Asp Glu Leu Leu Thr Val
755 760 765
Thr Gly Glu Ile Glu Pro Arg Ser Ala Glu Ile Thr Phe Tyr Ser Thr
770 775 780
Val Asp Val Arg Ala Val Asp Gly Thr Asp Leu Asp Ala Gly Tyr Trp
785 790 795 800
Tyr Arg Asn Leu Arg Glu Thr Val Arg Phe Ala Asp Ala Met Thr Arg
805 810 815
Leu Ala Asp Ser Gly Tyr Asp Ala Phe Val Glu Val Ser Pro His Pro
820 825 830
Val Val Val Ser Ala Val Ala Glu Ala Val Glu Glu Ala Gly Val Glu
835 840 845
Asp Ala Val Val Val Gly Thr Leu Ser Arg Gly Asp Gly Gly Pro Gly
850 855 860
Ala Phe Leu Arg Ser Ala Ala Thr Ala His Cys Ala Gly Val Asp Val
865 870 875 880
Asp Trp Thr Pro Ala Leu Pro Gly Ala Ala Thr Ile Pro Leu Pro Thr
885 890 895

Tyr Pro Phe Gln Arg Lys Pro Tyr Trp Leu Arg Ser Ser Ala Pro Ala
900 905 910
Pro Ala Ser His Asp Leu Ala Tyr Arg Val Ser Trp Thr Pro Ile Thr
915 920 925
Pro Pro Gly Asp Gly Val Leu Asp Gly Asp Trp Leu Val Val His Pro
930 935 940
Gly Gly Ser Thr Gly Trp Val Asp Gly Leu Ala Ala Ala Ile Thr Ala
945 950 955 960
Gly Gly Gly Arg Val Val Ala His Pro Val Asp Ser Val Thr Ser Arg
965 970 975
Thr Gly Leu Ala Glu Ala Leu Ala Arg Arg Asp Gly Thr Phe Arg Gly
980 985 990
Val Leu Ser Trp Val Ala Thr Asp Glu Arg His Val Glu Ala Gly Ala
995 1000 1005
Val Ala Leu Leu Thr Leu Ala Gln Ala Leu Gly Asp Ala Gly Ile Asp
1010 1015 1020
Ala Pro Leu Trp Cys Leu Thr Gln Glu Ala Val Arg Thr Pro Val Asp
1025 1030 1035 1040
Gly Asp Leu Ala Arg Pro Ala Gln Ala Ala Leu His Gly Phe Ala Gln
1045 1050 1055
Val Ala Arg Leu Glu Leu Ala Arg Arg Phe Gly Gly Val Leu Asp Leu
1060 1065 1070
Pro Ala Thr Val Asp Ala Ala Gly Thr Arg Leu Val Ala Ala Val Leu
1075 1080 1085
Ala Gly Gly Gly Glu Asp Val Val Ala Val Arg Gly Asp Arg Leu Tyr
1090 1095 1100
Gly Arg Arg Leu Val Arg Ala Thr Leu Pro Pro Pro Gly Gly Gly Phe
1105 1110 1115 1120
Thr Pro His Gly Thr Val Leu Val Thr Gly Ala Ala Gly Pro Val Gly
1125 1130 1135
Gly Arg Leu Ala Arg Trp Leu Ala Glu Arg Gly Ala Thr Arg Leu Val
1140 1145 1150
Leu Pro Gly Ala His Pro Gly Glu Glu Leu Leu Thr Ala Ile Arg Ala
1155 1160 1165
Ala Gly Ala Thr Ala Val Val Cys Glu Pro Glu Ala Glu Ala Leu Arg
1170 1175 1180
Thr Ala Ile Gly Gly Glu Leu Pro Thr Ala Leu Val His Ala Glu Thr
1185 1190 1195 1200
Leu Thr Asn Phe Ala Gly Val Ala Asp Ala Asp Pro Glu Asp Phe Ala
1205 1210 1215
Ala Thr Val Ala Ala Lys Thr Ala Leu Pro Thr Val Leu Ala Glu Val
1220 1225 1230
Leu Gly Asp His Arg Leu Glu Arg Glu Val Tyr Cys Ser Ser Val Ala
1235 1240 1245
Gly Val Trp Gly Gly Val Gly Met Ala Ala Tyr Ala Ala Gly Ser Ala
1250 1255 1260
Tyr Leu Asp Ala Leu Val Glu His Arg Arg Ala Arg Gly His Ala Ser
1265 1270 1275 1280
Ala Ser Val Ala Trp Thr Pro Trp Ala Leu Pro Gly Ala Val Asp Asp
1285 1290 1295
Gly Arg Leu Arg Glu Arg Gly Leu Arg Ser Leu Asp Val Ala Asp Ala
1300 1305 1310
Leu Gly Thr Trp Glu Arg Leu Leu Arg Ala Gly Ala Val Ser Val Ala
1315 1320 1325
Val Ala Asp Val Asp Trp Ser Val Phe Thr Glu Gly Phe Ala Ala Ile
1330 1335 1340
Arg Pro Thr Pro Leu Phe Asp Glu Leu Leu Asp Arg Arg Gly Asp Pro
1345 1350 1355 1360
Asp Gly Ala Pro Val Asp Arg Pro Gly Glu Pro Ala Gly Glu Trp Gly
1365 1370 1375
Arg Arg Ile Ala Ala Leu Ser Pro Gln Glu Gln Arg Glu Thr Leu Leu

1380 1385 1390
Thr Leu Val Gly Glu Thr Val Ala Glu Val Leu Gly His Glu Thr Gly
1395 1400 1405
Thr Glu Ile Asn Thr Arg Arg Ala Phe Ser Glu Leu Gly Leu Asp Ser
1410 1415 1420
Leu Gly Ser Met Ala Leu Arg Gln Arg Leu Ala Ala Arg Thr Gly Leu
1425 1430 1435 1440
Arg Met Pro Ala Ser Leu Val Phe Asp His Pro Thr Val Thr Ala Leu
1445 1450 1455
Ala Arg Tyr Leu Arg Arg Leu Val Val Gly Asp Ser Asp Pro Thr Pro
1460 1465 1470
Val Arg Val Phe Gly Pro Thr Asp Glu Ala Glu Pro Val Ala Val Val
1475 1480 1485
Gly Ile Gly Cys Arg Phe Pro Gly Gly Ile Ala Thr Pro Glu Asp Leu
1490 1495 1500
Trp Arg Val Val Ser Glu Gly Thr Ser Ile Thr Thr Gly Phe Pro Thr
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Asp Arg Gly Trp Asp Leu Arg Arg Leu Tyr His Pro Asp Pro Asp His
1525 1530 1535
Pro Gly Thr Ser Tyr Val Asp Arg Gly Gly Phe Leu Asp Gly Ala Pro
1540 1545 1550
Asp Phe Asp Pro Gly Phe Phe Gly Ile Thr Pro Arg Glu Ala Leu Ala
1555 1560 1565
Met Asp Pro Gln Gln Arg Leu Thr Leu Glu Ile Ala Trp Glu Ala Val
1570 1575 1580
Glu Arg Ala Gly Ile Asp Pro Glu Thr Leu Leu Gly Ser Asp Thr Gly
1585 1590 1595 1600
Val Phe Val Gly Met Asn Gly Gln Ser Tyr Leu Gln Leu Leu Thr Gly
1605 1610 1615
Glu Gly Asp Arg Leu Asn Gly Tyr Gln Gly Leu Gly Asn Ser Ala Ser
1620 1625 1630
Val Leu Ser Gly Arg Val Ala Tyr Thr Phe Gly Trp Glu Gly Pro Ala
1635 1640 1645
Leu Thr Val Asp Thr Ala Cys Ser Ser Ser Leu Val Ala Ile His Leu
1650 1655 1660
Ala Met Gln Ser Leu Arg Arg Gly Glu Cys Ser Leu Ala Leu Ala Gly
1665 1670 1675 1680
Gly Val Thr Val Met Ala Asp Pro Tyr Thr Phe Val Asp Phe Ser Ala
1685 1690 1695
Gln Arg Gly Leu Ala Ala Asp Gly Arg Cys Lys Ala Phe Ser Ala Gln
1700 1705 1710
Ala Asp Gly Phe Ala Leu Ala Glu Gly Val Ala Ala Leu Val Leu Glu
1715 1720 1725
Pro Leu Ser Lys Ala Arg Arg Asn Gly His Gln Val Leu Ala Val Leu
1730 1735 1740
Arg Gly Ser Ala Val Asn Gln Asp Gly Ala Ser Asn Gly Leu Ala Ala
1745 1750 1755 1760
Pro Asn Gly Pro Ser Gln Glu Arg Val Ile Arg Gln Ala Leu Thr Ala
1765 1770 1775
Ser Gly Leu Arg Pro Ala Asp Val Asp Met Val Glu Ala His Gly Thr
1780 1785 1790
Gly Thr Glu Leu Gly Asp Pro Ile Glu Ala Gly Ala Leu Ile Ala Ala
1795 1800 1805
Tyr Gly Arg Asp Arg Asp Arg Pro Leu Trp Leu Gly Ser Val Lys Thr
1810 1815 1820
Asn Ile Gly His Thr Gln Ala Ala Ala Gly Ala Ala Gly Val Ile Lys
1825 1830 1835 1840
Ala Val Leu Ala Met Arg His Gly Val Leu Pro Arg Ser Leu His Ala
1845 1850 1855
Asp Glu Leu Ser Pro His Ile Asp Trp Ala Asp Gly Lys Val Glu Val
1860 1865 1870

Leu Arg Glu Ala Arg Gln Trp Pro Pro Gly Glu Arg Pro Arg Arg Ala
1875 1880 1885
Gly Val Ser Ser Phe Gly Val Ser Gly Thr Asn Ala His Val Ile Val
1890 1895 1900
Glu Glu Ala Pro Ala Glu Pro Asp Pro Glu Pro Val Pro Ala Ala Pro
1905 1910 1915 1920
Gly Gly Pro Leu Pro Phe Val Leu His Gly Arg Ser Val Gln Thr Val
1925 1930 1935
Arg Ser Gln Ala Arg Thr Leu Ala Glu His Leu Arg Thr Thr Gly His
1940 1945 1950
Arg Asp Leu Ala Asp Thr Ala Arg Thr Leu Ala Thr Gly Arg Ala Arg
1955 1960 1965
Phe Asp Val Arg Ala Ala Val Leu Gly Thr Asp Arg Glu Gly Val Cys
1970 1975 1980
Ala Ala Leu Asp Ala Leu Ala Gln Asp Arg Pro Ser Pro Asp Val Val
1985 1990 1995 2000
Ala Pro Ala Val Phe Ala Ala Arg Thr Pro Val Leu Val Phe Pro Gly
2005 2010 2015
Gln Gly Ser Gln Trp Val Gly Met Ala Arg Asp Leu Leu Asp Ser Ser
2020 2025 2030
Glu Val Phe Ala Glu Ser Met Gly Arg Cys Ala Glu Ala Leu Ser Pro
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Tyr Thr Asp Trp Asp Leu Leu Asp Val Val Arg Gly Val Gly Asp Pro
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Met Val Ser Leu Ala Arg Leu Trp Gln Ser Tyr Gly Val Thr Pro Gly
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Ala Val Val Gly His Ser Gln Gly Glu Ile Ala Ala Ala His Val Ala
2100 2105 2110
Gly Ala Leu Ser Leu Ala Asp Ala Ala Arg Val Val Ala Leu Arg Ser
2115 2120 2125
Arg Val Leu Arg Glu Leu Asp Asp Gln Gly Gly Met Val Ser Val Gly
2130 2135 2140
Thr Ser Arg Ala Glu Leu Asp Ser Val Leu Arg Arg Trp Asp Gly Arg
2145 2150 2155 2160
Val Ala Val Ala Ala Val Asn Gly Pro Gly Thr Leu Val Val Ala Gly
2165 2170 2175
Pro Thr Ala Glu Leu Asp Glu Phe Leu Ala Val Ala Glu Ala Arg Glu
2180 2185 2190
Met Arg Pro Arg Arg Ile Ala Val Arg Tyr Ala Ser His Ser Pro Glu
2195 2200 2205
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2225 2230 2235 2240
Leu Asp Thr Thr Ala Met Asp Ala Gly Tyr Trp Tyr Arg Asn Leu Arg
2245 2250 2255
Gln Pro Val Leu Phe Glu His Ala Val Arg Ser Leu Leu Glu Arg Gly
2260 2265 2270
Phe Glu Thr Phe Ile Glu Val Ser Pro His Pro Val Leu Leu Met Ala
2275 2280 2285
Val Glu Glu Thr Ala Glu Asp Ala Glu Arg Pro Val Thr Gly Val Pro
2290 2295 2300
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Leu Gly Ala His Val His Gly Val Asp Val Asp Leu Arg Pro Ala Val
2325 2330 2335
Ala His Gly Arg Leu Val Asp Leu Pro Thr Tyr Pro Phe Asp Arg Gln
2340 2345 2350
Arg Leu Trp Pro Lys Pro His Arg Arg Ala Asp Thr Ser Ser Leu Gly

2355 2360 2365
Val Arg Asp Ser Thr His Pro Leu Leu His Ala Ala Val Asp Val Pro
2370 2375 2380
Gly His Gly Gly Ala Val Phe Thr Gly Arg Leu Ser Pro Asp Glu Gln
2385 2390 2395 2400
Gln Trp Leu Thr Gln His Val Val Gly Gly Arg Asn Leu Val Pro Gly
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2420 2425 2430
Pro Val Leu Glu Glu Leu Val Leu Gln Gln Pro Leu Val Leu Thr Ala
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Ala Gly Ala Leu Leu Arg Leu Ser Val Gly Ala Ala Asp Glu Asp Gly
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Ala Gly Gly Gly Arg Asp Gly Thr Gln Trp Pro Pro Pro Gly Ala Thr
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Glu Tyr Gly Pro Ala Phe Gln Ala Leu Arg Ala Ala Trp Gln His Gly
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Asp Val Val Tyr Ala Glu Val Ser Leu Asp Ala Val Glu Glu Gly Tyr
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Ala Phe Asp Pro Val Leu Leu Asp Ala Val Ala Gln Thr Phe Gly Leu
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Thr Ser Arg Ala Pro Gly Lys Leu Pro Phe Ala Trp Arg Gly Val Thr
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Leu His Ala Thr Gly Ala Thr Ala Val Arg Val Val Ala Thr Pro Ala
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2625 2630 2635 2640
Asp Gln Pro Arg Gly Arg Asp Gly Asp Leu His Arg Leu Glu Trp Val
2645 2650 2655
Arg Leu Ala Thr Pro Asp Pro Thr Pro Ala Ala Val Val His Val Ala
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Ala Asp Gly Leu Asp Asp Leu Leu Arg Ala Gly Gly Pro Ala Pro Gln
2675 2680 2685
Ala Val Val Val Arg Tyr Arg Pro Asp Gly Asp Asp Pro Thr Ala Glu
2690 2695 2700
Ala Arg His Gly Val Leu Trp Ala Ala Thr Leu Val Arg Arg Trp Leu
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Asp Asp Asp Arg Trp Pro Ala Thr Thr Leu Val Val Ala Thr Ser Ala
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Gly Val Glu Val Ser Pro Gly Asp Asp Val Pro Arg Pro Gly Ala Ala
2740 2745 2750
Ala Val Trp Gly Val Leu Arg Cys Ala Gln Ala Glu Ser Pro Asp Arg
2755 2760 2765
Phe Val Leu Val Asp Gly Asp Pro Glu Thr Pro Pro Ala Val Pro Asp
2770 2775 2780
Asn Pro Gln Leu Ala Val Arg Asp Gly Ala Val Phe Val Pro Arg Leu
2785 2790 2795 2800
Thr Pro Leu Ala Gly Pro Val Pro Ala Val Ala Asp Arg Ala Tyr Arg
2805 2810 2815
Leu Val Pro Gly Asn Gly Gly Ser Ile Glu Ala Val Ala Phe Ala Pro
2820 2825 2830
Val Pro Asp Ala Asp Arg Pro Leu Ala Pro Glu Glu Val Arg Val Ala
2835 2840 2845

Val Arg Ala Thr Gly Val Asn Phe Arg Asp Val Leu Leu Ala Leu Gly
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Met Tyr Pro Glu Pro Ala Glu Met Gly Thr Glu Ala Ser Gly Val Val
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Val Phe Ala Thr Ala Ser Pro Ala Lys His Pro Thr Leu Arg Ala Leu
2980 2985 2990
Gly Leu Asp Asp Asp His Ile Ala Ser Ser Arg Glu Ser Gly Phe Gly
2995 3000 3005
Glu Arg Phe Ala Ala Arg Thr Gly Gly Arg Gly Val Asp Val Val Leu
3010 3015 3020
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Glu Gln Phe Arg Gly Arg Tyr Val Pro Phe Asp Leu Ala Glu Ala Gly
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Pro Asp Arg Leu Gly Glu Ile Leu Glu Glu Val Val Gly Leu Leu Ala
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Ala Pro Ala Ala Leu Thr His Met Ser Arg Gly Arg His Val Gly Lys
3105 3110 3115 3120
Leu Val Leu Thr Gln Pro Ala Pro Val His Pro Asp Gly Thr Val Leu
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Val Thr Gly Gly Thr Gly Thr Leu Gly Arg Leu Val Ala Arg His Leu
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Val Thr Gly His Gly Val Pro His Leu Leu Val Ala Ser Arg Arg Gly
3155 3160 3165
Pro Ala Ala Pro Gly Ala Ala Glu Leu Arg Ala Asp Val Glu Gly Leu
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Gly Ala Thr Ile Glu Ile Val Ala Cys Asp Thr Ala Asp Arg Glu Ala
3185 3190 3195 3200
Leu Ala Ala Leu Leu Asp Ser Ile Pro Ala Asp Arg Pro Leu Thr Gly
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Val Val His Thr Ala Gly Val Leu Ala Asp Gly Leu Val Thr Ser Ile
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Asp Gly Thr Ala Thr Asp Gln Val Leu Arg Ala Lys Val Asp Ala Ala
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Trp His Leu His Asp Leu Thr Arg Asp Ala Asp Leu Ser Phe Phe Val
3250 3255 3260
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3265 3270 3275 3280
Tyr Ala Ala Ala Asn Gly Val Leu Asn Ala Leu Ala Gly Gln Arg Arg
3285 3290 3295
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3315 3320 3325
Val Ala Ala Leu Pro Thr Glu Arg Ala Leu Ala Leu Phe Asp Ala Ala

3330 3335 3340
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Ala Leu Arg Arg Ala Glu Tyr Val Pro Glu Val Leu Arg Gly Ala Val
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Arg Ser Thr Pro Arg Ala Ala Asn Arg Ala Glu Thr Pro Gly Arg Gly
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Ser Ala Asp Gln Leu Pro Glu Arg Lys Ala Phe Lys Asp Leu Gly Phe
3425 3430 3435 3440
Asp Ser Leu Ala Ala Val Glu Leu Arg Asn Arg Leu Gly Val Thr Thr
3445 3450 3455
Gly Val Arg Leu Pro Ser Thr Leu Val Phe Asp His Pro Thr Pro Leu
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Ala Val Ala Glu His Leu Arg Ser Glu Leu Phe Ala Asp Ser Ala Pro
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Asp Val Gly Val Gly Ala Arg Leu Asp Asp Leu Glu Arg Ala Leu Asp
3490 3495 3500
Ala Leu Pro Asp Ala Gln Gly His Ala Asp Val Gly Ala Arg Leu Glu
3505 3510 3515 3520
Ala Leu Leu Arg Arg Trp Gln Ser Arg Arg Pro Pro Glu Thr Glu Pro
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35 40 45
Arg Phe Pro Gly Gly Val Asp Ser Pro Glu Ala Phe Trp Glu Phe Ile
50 55 60
Arg Asp Gly Gly Asp Ala Ile Ala Glu Ala Pro Thr Asp Arg Gly Trp
65 70 75 80
Pro Pro Ala Pro Arg Pro Arg Leu Gly Gly Leu Leu Ala Glu Pro Gly
85 90 95
Ala Phe Asp Ala Ala Phe Phe Gly Ile Ser Pro Arg Glu Ala Leu Ala
100 105 110
Thr Asp Pro Gln Gln Arg Leu Met Leu Glu Ile Ser Trp Glu Ala Leu
115 120 125
Glu Arg Ala Gly Phe Asp Pro Ser Ser Leu Arg Gly Ser Ala Gly Gly
130 135 140
Val Phe Thr Gly Val Gly Ala Val Asp Tyr Gly Pro Arg Pro Asp Glu
145 150 155 160
Ala Pro Glu Glu Val Leu Gly Tyr Val Gly Ile Gly Thr Ala Ser Ser
165 170 175
Val Ala Ser Gly Arg Val Ala Tyr Thr Leu Gly Leu Glu Gly Pro Ala
180 185 190
Val Thr Val Asp Thr Ala Cys Ser Ser Gly Leu Thr Ala Val His Leu

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Ala Met Glu Ser Leu Arg Arg Asp Glu Cys Thr Leu Val Leu Ala Gly
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Gly Val Thr Val Met Ser Ser Pro Gly Ala Phe Thr Glu Phe Arg Ser
225                 230                 235                 240
Gln Gly Gly Leu Ala Glu Asp Gly Arg Cys Lys Pro Phe Ser Arg Ala
                245                 250                 255
Ala Asp Gly Phe Gly Leu Ala Glu Gly Ala Gly Val Leu Val Leu Gln
            260                 265                 270
Arg Leu Ser Val Ala Arg Ala Glu Gly Arg Pro Val Leu Ala Val Leu
        275                 280                 285
Arg Gly Ser Ala Ile Asn Gln Asp Gly Ala Ser Asn Gly Leu Thr Ala
    290                 295                 300
Pro Ser Gly Pro Ala Gln Arg Arg Val Ile Arg Gln Ala Leu Glu Arg
305                 310                 315                 320
Ala Arg Leu Arg Pro Val Asp Val Asp Tyr Val Glu Ala His Gly Thr
                325                 330                 335
Gly Thr Arg Leu Gly Asp Pro Ile Glu Ala His Ala Leu Leu Asp Thr
            340                 345                 350
Tyr Gly Ala Asp Arg Glu Pro Gly Arg Pro Leu Trp Val Gly Ser Val
        355                 360                 365
Lys Ser Asn Ile Gly His Thr Gln Ala Ala Ala Gly Val Ala Gly Val
    370                 375                 380
Met Lys Thr Val Leu Ala Leu Arg His Arg Glu Ile Pro Ala Thr Leu
385                 390                 395                 400
His Phe Asp Glu Pro Ser Pro His Val Asp Trp Asp Arg Gly Ala Val
                405                 410                 415
Ser Val Val Ser Glu Thr Arg Pro Trp Pro Val Gly Glu Arg Pro Arg
            420                 425                 430
Arg Ala Gly Val Ser Ser Phe Gly Ile Ser Gly Thr Asn Ala His Val
        435                 440                 445
Ile Val Glu Glu Ala Pro Ser Pro Gln Ala Ala Asp Leu Asp Pro Thr
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Pro Gly Pro Ala Thr Gly Ala Thr Pro Gly Thr Asp Ala Ala Pro Thr
465                 470                 475                 480
Ala Glu Pro Gly Ala Glu Ala Val Ala Leu Val Phe Ser Ala Arg Asp
                485                 490                 495
Glu Arg Ala Leu Arg Ala Gln Ala Ala Arg Leu Ala Asp Arg Leu Thr
            500                 505                 510
Asp Asp Pro Ala Pro Ser Leu Arg Asp Thr Ala Phe Thr Leu Val Thr
        515                 520                 525
Arg Arg Ala Thr Trp Glu His Arg Ala Val Val Val Gly Gly Gly Glu
    530                 535                 540
Glu Val Leu Ala Gly Leu Arg Ala Val Ala Gly Gly Arg Pro Val Asp
545                 550                 555                 560
Gly Ala Val Ser Gly Arg Ala Arg Ala Gly Arg Arg Val Val Leu Val
                565                 570                 575
Phe Pro Gly Gln Gly Ala Gln Trp Gln Gly Met Ala Arg Asp Leu Leu
            580                 585                 590
Arg Gln Ser Pro Thr Phe Ala Glu Ser Ile Asp Ala Cys Glu Arg Ala
        595                 600                 605
Leu Ala Pro His Val Asp Trp Ser Leu Arg Glu Val Leu Asp Gly Glu
    610                 615                 620
Gln Ser Leu Asp Pro Val Asp Val Val Gln Pro Val Leu Phe Ala Val
625                 630                 635                 640
Met Val Ser Leu Ala Arg Leu Trp Gln Ser Tyr Gly Val Thr Pro Gly
                645                 650                 655
Ala Val Val Gly His Ser Gln Gly Glu Ile Ala Ala Ala His Val Ala
            660                 665                 670
Gly Ala Leu Ser Leu Ala Asp Ala Ala Arg Val Val Ala Leu Arg Ser
        675                 680                 685
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Arg Val Leu Arg Arg Leu Gly Gly His Gly Gly Met Ala Ser Phe Gly
690 695 700
Leu His Pro Asp Gln Ala Ala Glu Arg Ile Ala Arg Phe Ala Gly Ala
705 710 715 720
Leu Thr Val Ala Ser Val Asn Gly Pro Arg Ser Val Val Leu Ala Gly
725 730 735
Glu Asn Gly Pro Leu Asp Glu Leu Ile Ala Glu Cys Glu Ala Glu Gly
740 745 750
Val Thr Ala Arg Arg Ile Pro Val Asp Tyr Ala Ser His Ser Pro Gln
755 760 765
Val Glu Ser Leu Arg Glu Glu Leu Leu Ala Ala Leu Ala Gly Val Arg
770 775 780
Pro Val Ser Ala Gly Ile Pro Leu Tyr Ser Thr Leu Thr Gly Gln Val
785 790 795 800
Ile Glu Thr Ala Thr Met Asp Ala Asp Tyr Trp Phe Ala Asn Leu Arg
805 810 815
Glu Pro Val Arg Phe Gln Asp Ala Thr Arg Gln Leu Ala Glu Ala Gly
820 825 830
Phe Asp Ala Phe Val Glu Val Ser Pro His Pro Val Leu Thr Val Gly
835 840 845
Val Glu Ala Thr Leu Glu Ala Val Leu Pro Pro Asp Ala Asp Pro Cys
850 855 860
Val Thr Gly Thr Leu Arg Arg Glu Arg Gly Gly Leu Ala Gln Phe His
865 870 875 880
Thr Ala Leu Ala Glu Ala Tyr Thr Arg Gly Val Glu Val Asp Trp Arg
885 890 895
Thr Ala Val Gly Glu Gly Arg Pro Val Asp Leu Pro Val Tyr Pro Phe
900 905 910
Gln Arg Gln Asn Phe Trp Leu Pro Val Pro Leu Gly Arg Val Pro Asp
915 920 925
Thr Gly Asp Glu Trp Arg Tyr Gln Leu Ala Trp His Pro Val Asp Leu
930 935 940
Gly Arg Ser Ser Leu Ala Gly Arg Val Leu Val Val Thr Gly Ala Ala
945 950 955 960
Val Pro Pro Ala Trp Thr Asp Val Val Arg Asp Gly Leu Glu Gln Arg
965 970 975
Gly Ala Thr Val Val Leu Cys Thr Ala Gln Ser Arg Ala Arg Ile Gly
980 985 990
Ala Ala Leu Asp Ala Val Asp Gly Thr Ala Leu Ser Thr Val Val Ser
995 1000 1005
Leu Leu Ala Leu Ala Glu Gly Gly Ala Val Asp Asp Pro Ser Leu Asp
1010 1015 1020
Thr Leu Ala Leu Val Gln Ala Leu Gly Ala Ala Gly Ile Asp Val Pro
1025 1030 1035 1040
Leu Trp Leu Val Thr Arg Asp Ala Ala Ala Val Thr Val Gly Asp Asp
1045 1050 1055
Val Asp Pro Ala Gln Ala Met Val Gly Gly Leu Gly Arg Val Val Gly
1060 1065 1070
Val Glu Ser Pro Ala Arg Trp Gly Gly Leu Val Asp Leu Arg Glu Ala
1075 1080 1085
Asp Ala Asp Ser Ala Arg Ser Leu Ala Ala Ile Leu Ala Asp Pro Arg
1090 1095 1100
Gly Glu Glu Gln Phe Ala Ile Arg Pro Asp Gly Val Thr Val Ala Arg
1105 1110 1115 1120
Leu Val Pro Ala Pro Ala Arg Ala Ala Gly Thr Arg Trp Thr Pro Arg
1125 1130 1135
Gly Thr Val Leu Val Thr Gly Gly Thr Gly Gly Ile Gly Ala His Leu
1140 1145 1150
Ala Arg Trp Leu Ala Gly Ala Gly Ala Glu His Leu Val Leu Leu Asn
1155 1160 1165
Arg Arg Gly Ala Glu Ala Ala Gly Ala Ala Asp Leu Arg Asp Glu Leu

1170 1175 1180
Val Ala Leu Gly Thr Gly Val Thr Ile Thr Ala Cys Asp Val Ala Asp
1185 1190 1195 1200
Arg Asp Arg Leu Ala Ala Val Leu Asp Ala Ala Arg Ala Gln Gly Arg
1205 1210 1215
Val Val Thr Ala Val Phe His Ala Ala Gly Ile Ser Arg Ser Thr Ala
1220 1225 1230
Val Gln Glu Leu Thr Glu Ser Glu Phe Thr Glu Ile Thr Asp Ala Lys
1235 1240 1245
Val Arg Gly Thr Ala Asn Leu Ala Glu Leu Cys Pro Glu Leu Asp Ala
1250 1255 1260
Leu Val Leu Phe Ser Ser Asn Ala Ala Val Trp Gly Ser Pro Gly Leu
1265 1270 1275 1280
Ala Ser Tyr Ala Ala Gly Asn Ala Phe Leu Asp Ala Phe Ala Arg Arg
1285 1290 1295
Gly Arg Arg Ser Gly Leu Pro Val Thr Ser Ile Ala Trp Gly Leu Trp
1300 1305 1310
Ala Gly Gln Asn Met Ala Gly Thr Glu Gly Gly Asp Tyr Leu Arg Ser
1315 1320 1325
Gln Gly Leu Arg Ala Met Asp Pro Gln Arg Ala Ile Glu Glu Leu Arg
1330 1335 1340
Thr Thr Leu Asp Ala Gly Asp Pro Trp Val Ser Val Val Asp Leu Asp
1345 1350 1355 1360
Arg Glu Arg Phe Val Glu Leu Phe Thr Ala Ala Arg Arg Arg Pro Leu
1365 1370 1375
Phe Asp Glu Leu Gly Gly Val Arg Ala Gly Ala Glu Glu Thr Gly Gln
1380 1385 1390
Glu Ser Asp Leu Ala Arg Arg Leu Ala Ser Met Pro Glu Ala Glu Arg
1395 1400 1405
His Glu His Val Ala Arg Leu Val Arg Ala Glu Val Ala Ala Val Leu
1410 1415 1420
Gly His Gly Thr Pro Thr Val Ile Glu Arg Asp Val Ala Phe Arg Asp
1425 1430 1435 1440
Leu Gly Phe Asp Ser Met Thr Ala Val Asp Leu Arg Asn Arg Leu Ala
1445 1450 1455
Ala Val Thr Gly Val Arg Val Ala Thr Thr Ile Val Phe Asp His Pro
1460 1465 1470
Thr Val Asp Arg Leu Thr Ala His Tyr Leu Glu Arg Leu Val Gly Glu
1475 1480 1485
Pro Glu Ala Thr Thr Pro Ala Ala Ala Val Val Pro Gln Ala Pro Gly
1490 1495 1500
Glu Ala Asp Glu Pro Ile Ala Ile Val Gly Met Ala Cys Arg Leu Ala
1505 1510 1515 1520
Gly Gly Val Arg Thr Pro Asp Gln Leu Trp Asp Phe Ile Val Ala Asp
1525 1530 1535
Gly Asp Ala Val Thr Glu Met Pro Ser Asp Arg Ser Trp Asp Leu Asp
1540 1545 1550
Ala Leu Phe Asp Pro Asp Pro Glu Arg His Gly Thr Ser Tyr Ser Arg
1555 1560 1565
His Gly Ala Phe Leu Asp Gly Ala Ala Asp Phe Asp Ala Ala Phe Phe
1570 1575 1580
Gly Ile Ser Pro Arg Glu Ala Leu Ala Met Asp Pro Gln Gln Arg Gln
1585 1590 1595 1600
Val Leu Glu Thr Thr Trp Glu Leu Phe Glu Asn Ala Gly Ile Asp Pro
1605 1610 1615
His Ser Leu Arg Gly Thr Asp Thr Gly Val Phe Leu Gly Ala Ala Tyr
1620 1625 1630
Gln Gly Tyr Gly Gln Asn Ala Gln Val Pro Lys Glu Ser Glu Gly Tyr
1635 1640 1645
Leu Leu Thr Gly Gly Ser Ser Ala Val Ala Ser Gly Arg Ile Ala Tyr
1650 1655 1660

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Val Leu Gly Leu Glu Gly Pro Ala Ile Thr Val Asp Thr Ala Cys Ser
1665                1670                1675                1680
Ser Ser Leu Val Ala Leu His Val Ala Ala Gly Ser Leu Arg Ser Gly
                1685                1690                1695
Asp Cys Gly Leu Ala Val Ala Gly Gly Val Ser Val Met Ala Gly Pro
            1700                1705                1710
Glu Val Phe Thr Glu Phe Ser Arg Gln Gly Ala Leu Ala Pro Asp Gly
        1715                1720                1725
Arg Cys Lys Pro Phe Ser Asp Gln Ala Asp Gly Phe Gly Phe Ala Glu
    1730                1735                1740
Gly Val Ala Val Val Leu Leu Gln Arg Leu Ser Val Ala Val Arg Glu
1745                1750                1755                1760
Gly Arg Arg Val Leu Gly Val Val Val Gly Ser Ala Val Asn Gln Asp
                1765                1770                1775
Gly Ala Ser Asn Gly Leu Ala Ala Pro Ser Gly Val Ala Gln Gln Arg
            1780                1785                1790
Val Ile Arg Arg Ala Trp Gly Arg Ala Gly Val Ser Gly Gly Asp Val
        1795                1800                1805
Gly Val Val Glu Ala His Gly Thr Gly Thr Arg Leu Gly Asp Pro Val
    1810                1815                1820
Glu Leu Gly Ala Leu Leu Gly Thr Tyr Gly Val Gly Arg Gly Gly Val
1825                1830                1835                1840
Gly Pro Val Val Val Gly Ser Val Lys Ala Asn Val Gly His Val Gln
                1845                1850                1855
Ala Ala Ala Gly Val Val Gly Val Ile Lys Val Val Leu Gly Leu Gly
            1860                1865                1870
Arg Gly Leu Val Gly Pro Met Val Cys Arg Gly Gly Leu Ser Gly Leu
        1875                1880                1885
Val Asp Trp Ser Ser Gly Gly Leu Val Val Ala Asp Gly Val Arg Gly
    1890                1895                1900
Trp Pro Val Gly Val Asp Gly Val Arg Arg Gly Gly Val Ser Ala Phe
1905                1910                1915                1920
Gly Val Ser Gly Thr Asn Ala His Val Val Val Ala Glu Ala Pro Gly
                1925                1930                1935
Ser Val Val Gly Ala Glu Arg Pro Val Glu Gly Ser Ser Arg Gly Leu
            1940                1945                1950
Val Gly Val Ala Gly Gly Val Val Pro Val Val Leu Ser Ala Lys Thr
        1955                1960                1965
Glu Thr Ala Leu Thr Glu Leu Ala Arg Arg Leu His Asp Ala Val Asp
    1970                1975                1980
Asp Thr Val Ala Leu Pro Ala Val Ala Ala Thr Leu Ala Thr Gly Arg
1985                1990                1995                2000
Ala His Leu Pro Tyr Arg Ala Ala Leu Leu Ala Arg Asp His Asp Glu
                2005                2010                2015
Leu Arg Asp Arg Leu Arg Ala Phe Thr Thr Gly Ser Ala Ala Pro Gly
            2020                2025                2030
Val Val Ser Gly Val Ala Ser Gly Gly Gly Val Val Phe Val Phe Pro
        2035                2040                2045
Gly Gln Gly Gly Gln Trp Val Gly Met Ala Arg Gly Leu Leu Ser Val
    2050                2055                2060
Pro Val Phe Val Glu Ser Val Val Glu Cys Asp Ala Val Val Ser Ser
2065                2070                2075                2080
Val Val Gly Phe Ser Val Leu Gly Val Leu Glu Gly Arg Ser Gly Ala
                2085                2090                2095
Pro Ser Leu Asp Arg Val Asp Val Val Gln Pro Val Leu Phe Val Val
            2100                2105                2110
Met Val Ser Leu Ala Arg Leu Trp Arg Trp Cys Gly Val Val Pro Ala
        2115                2120                2125
Ala Val Val Gly His Ser Gln Gly Glu Ile Ala Ala Ala Val Val Ala
    2130                2135                2140
Gly Val Leu Ser Val Gly Asp Gly Ala Arg Val Val Ala Leu Arg Ala
```

2145 2150 2155 2160
Arg Ala Leu Arg Ala Leu Ala Gly His Gly Gly Met Val Ser Leu Ala
2165 2170 2175
Val Ser Ala Glu Arg Ala Arg Glu Leu Ile Ala Pro Trp Ser Asp Arg
2180 2185 2190
Ile Ser Val Ala Ala Val Asn Ser Pro Thr Ser Val Val Val Ser Gly
2195 2200 2205
Asp Pro Gln Ala Leu Ala Ala Leu Val Ala His Cys Ala Glu Thr Gly
2210 2215 2220
Glu Arg Ala Lys Thr Leu Pro Val Asp Tyr Ala Ser His Ser Ala His
2225 2230 2235 2240
Val Glu Gln Ile Arg Asp Thr Ile Leu Thr Asp Leu Ala Asp Val Thr
2245 2250 2255
Ala Arg Arg Pro Asp Val Ala Leu Tyr Ser Thr Leu His Gly Ala Arg
2260 2265 2270
Gly Ala Gly Thr Asp Met Asp Ala Arg Tyr Trp Tyr Asp Asn Leu Arg
2275 2280 2285
Ser Pro Val Arg Phe Asp Glu Ala Val Glu Ala Ala Val Ala Asp Gly
2290 2295 2300
Tyr Arg Val Phe Val Glu Met Ser Pro His Pro Val Leu Thr Ala Ala
2305 2310 2315 2320
Val Gln Glu Ile Asp Asp Glu Thr Val Ala Ile Gly Ser Leu His Arg
2325 2330 2335
Asp Thr Gly Glu Arg His Leu Val Ala Glu Leu Ala Arg Ala His Val
2340 2345 2350
His Gly Val Pro Val Asp Trp Arg Ala Ile Leu Pro Ala Thr His Pro
2355 2360 2365
Val Pro Leu Pro Asn Tyr Pro Phe Glu Ala Thr Arg Tyr Trp Leu Ala
2370 2375 2380
Pro Thr Ala Ala Asp Gln Val Ala Asp His Arg Tyr Arg Val Asp Trp
2385 2390 2395 2400
Arg Pro Leu Ala Thr Thr Pro Ala Glu Leu Ser Gly Ser Tyr Leu Val
2405 2410 2415
Phe Gly Asp Ala Pro Glu Thr Leu Gly His Ser Val Glu Lys Ala Gly
2420 2425 2430
Gly Leu Leu Val Pro Val Ala Ala Pro Asp Arg Glu Ser Leu Ala Val
2435 2440 2445
Ala Leu Asp Glu Ala Ala Gly Arg Leu Ala Gly Val Leu Ser Phe Ala
2450 2455 2460
Ala Asp Thr Ala Thr His Leu Ala Arg His Arg Leu Leu Gly Glu Ala
2465 2470 2475 2480
Asp Val Glu Ala Pro Leu Trp Leu Val Thr Ser Gly Gly Val Ala Leu
2485 2490 2495
Asp Asp His Asp Pro Ile Asp Cys Asp Gln Ala Met Val Trp Gly Ile
2500 2505 2510
Gly Arg Val Met Gly Leu Glu Thr Pro His Arg Trp Gly Gly Leu Val
2515 2520 2525
Asp Val Thr Val Glu Pro Thr Ala Glu Asp Gly Val Val Phe Ala Ala
2530 2535 2540
Leu Leu Ala Ala Asp Asp His Glu Asp Gln Val Ala Leu Arg Asp Gly
2545 2550 2555 2560
Ile Arg His Gly Arg Arg Leu Val Arg Ala Pro Leu Thr Thr Arg Asn
2565 2570 2575
Ala Arg Trp Thr Pro Ala Gly Thr Ala Leu Val Thr Gly Gly Thr Gly
2580 2585 2590
Ala Leu Gly Gly His Val Ala Arg Tyr Leu Ala Arg Ser Gly Val Thr
2595 2600 2605
Asp Leu Val Leu Leu Ser Arg Ser Gly Pro Asp Ala Pro Gly Ala Ala
2610 2615 2620
Glu Leu Ala Ala Glu Leu Ala Asp Leu Gly Ala Glu Pro Arg Val Glu
2625 2630 2635 2640

Ala Cys Asp Val Thr Asp Gly Pro Arg Leu Arg Ala Leu Val Gln Glu
2645 2650 2655
Leu Arg Glu Gln Asp Arg Pro Val Arg Ile Val Val His Thr Ala Gly
2660 2665 2670
Val Pro Asp Ser Arg Pro Leu Asp Arg Ile Asp Glu Leu Glu Ser Val
2675 2680 2685
Ser Ala Ala Lys Val Thr Gly Ala Arg Leu Leu Asp Glu Leu Cys Pro
2690 2695 2700
Asp Ala Asp Thr Phe Val Leu Phe Ser Ser Gly Ala Gly Val Trp Gly
2705 2710 2715 2720
Ser Ala Asn Leu Gly Ala Tyr Ala Ala Ala Asn Ala Tyr Leu Asp Ala
2725 2730 2735
Leu Ala His Arg Arg Arg Gln Ala Gly Arg Ala Ala Thr Ser Val Ala
2740 2745 2750
Trp Gly Ala Trp Ala Gly Asp Gly Met Ala Thr Gly Asp Leu Asp Gly
2755 2760 2765
Leu Thr Arg Arg Gly Leu Arg Ala Met Ala Pro Asp Arg Ala Leu Arg
2770 2775 2780
Ala Cys Thr Arg Arg Trp Thr Thr His Asp Thr Cys Val Ser Val Ala
2785 2790 2795 2800
Asp Val Asp Trp Asp Arg Phe Ala Val Gly Phe Thr Ala Ala Arg Pro
2805 2810 2815
Arg Pro Leu Ile Asp Glu Leu Val Thr Ser Ala Pro Val Ala Ala Pro
2820 2825 2830
Thr Ala Ala Ala Ala Pro Val Pro Ala Met Thr Ala Asp Gln Leu Leu
2835 2840 2845
Gln Phe Thr Arg Ser His Val Ala Ala Ile Leu Gly His Gln Asp Pro
2850 2855 2860
Asp Ala Val Gly Leu Asp Gln Pro Phe Thr Glu Leu Gly Phe Asp Ser
2865 2870 2875 2880
Leu Thr Ala Val Gly Leu Arg Asn Gln Leu Gln Gln Ala Thr Gly Arg
2885 2890 2895
Thr Leu Pro Ala Ala Leu Val Phe Gln His Pro Thr Val Arg Arg Leu
2900 2905 2910
Ala Asp His Leu Ala Gln Gln Leu Asp Val Gly Thr Ala Pro Val Glu
2915 2920 2925
Ala Thr Gly Ser Val Leu Arg Asp Gly Tyr Arg Arg Ala Gly Gln Thr
2930 2935 2940
Gly Asp Val Arg Ser Tyr Leu Asp Leu Leu Ala Asn Leu Ser Glu Phe
2945 2950 2955 2960
Arg Glu Arg Phe Thr Asp Ala Ala Ser Leu Gly Gly Gln Leu Glu Leu
2965 2970 2975
Val Asp Leu Ala Asp Gly Ser Gly Pro Val Thr Val Ile Cys Cys Ala
2980 2985 2990
Gly Thr Ala Ala Leu Ser Gly Pro His Glu Phe Ala Arg Leu Ala Ser
2995 3000 3005
Ala Leu Arg Gly Thr Val Pro Val Arg Ala Leu Ala Gln Pro Gly Tyr
3010 3015 3020
Glu Ala Gly Glu Pro Val Pro Ala Ser Met Glu Ala Val Leu Gly Val
3025 3030 3035 3040
Gln Ala Asp Ala Val Leu Ala Ala Gln Gly Asp Thr Pro Phe Val Leu
3045 3050 3055
Val Gly His Ser Ala Gly Ala Leu Met Ala Tyr Ala Leu Ala Thr Glu
3060 3065 3070
Leu Ala Asp Arg Gly His Pro Pro Arg Gly Val Val Leu Leu Asp Val
3075 3080 3085
Tyr Pro Pro Gly His Gln Glu Ala Val His Ala Trp Leu Gly Glu Leu
3090 3095 3100
Thr Ala Ala Leu Phe Asp His Glu Thr Val Arg Met Asp Asp Thr Arg
3105 3110 3115 3120
Leu Thr Ala Leu Gly Ala Tyr Asp Arg Leu Thr Gly Arg Trp Arg Pro

```
                3125                    3130                    3135
Arg Asp Thr Gly Leu Pro Thr Leu Val Val Ala Ala Ser Glu Pro Met
            3140                    3145                    3150
Gly Glu Trp Pro Asp Asp Gly Trp Gln Ser Thr Trp Pro Phe Gly His
        3155                    3160                    3165
Asp Arg Val Thr Val Pro Gly Asp His Phe Ser Met Val Gln Glu His
    3170                    3175                    3180
Ala Asp Ala Ile Ala Arg His Ile Asp Ala Trp Leu Ser Gly Glu Arg
3185                    3190                    3195                    3200
Ala
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<210> 16
<211> 358
<212> PRT
<213> Micromonospora megalomicea

<400> 16

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Met Asn Thr Thr Asp Arg Ala Val Leu Gly Arg Arg Leu Gln Met Ile
 1               5                   10                  15
Arg Gly Leu Tyr Trp Gly Tyr Gly Ser Asn Gly Asp Pro Tyr Pro Met
            20                  25                  30
Leu Leu Cys Gly His Asp Asp Asp Pro His Arg Trp Tyr Arg Gly Leu
        35                  40                  45
Gly Gly Ser Gly Val Arg Arg Ser Arg Thr Glu Thr Trp Val Val Thr
    50                  55                  60
Asp His Ala Thr Ala Val Arg Val Leu Asp Asp Pro Thr Phe Thr Arg
65                  70                  75                  80
Ala Thr Gly Arg Thr Pro Glu Trp Met Arg Ala Ala Gly Ala Pro Ala
                85                  90                  95
Ser Thr Trp Ala Gln Pro Phe Arg Asp Val His Ala Ala Ser Trp Asp
            100                 105                 110
Ala Glu Leu Pro Asp Pro Gln Glu Val Glu Asp Arg Leu Thr Gly Leu
        115                 120                 125
Leu Pro Ala Pro Gly Thr Arg Leu Asp Leu Val Arg Asp Leu Ala Trp
    130                 135                 140
Pro Met Ala Ser Arg Gly Val Gly Ala Asp Asp Pro Asp Val Leu Arg
145                 150                 155                 160
Ala Ala Trp Asp Ala Arg Val Gly Leu Asp Ala Gln Leu Thr Pro Gln
                165                 170                 175
Pro Leu Ala Val Thr Glu Ala Ala Ile Ala Ala Val Pro Gly Asp Pro
            180                 185                 190
His Arg Arg Ala Leu Phe Thr Ala Val Glu Met Thr Ala Thr Ala Phe
        195                 200                 205
Val Asp Ala Val Leu Ala Val Thr Ala Thr Ala Gly Ala Ala Gln Arg
    210                 215                 220
Leu Ala Asp Asp Pro Asp Val Ala Ala Arg Leu Val Ala Glu Val Leu
225                 230                 235                 240
Arg Leu His Pro Thr Ala His Leu Glu Arg Arg Thr Ala Gly Thr Glu
                245                 250                 255
Thr Val Val Gly Glu His Thr Val Ala Ala Gly Asp Glu Val Val Val
            260                 265                 270
Val Val Ala Ala Ala Asn Arg Asp Ala Gly Val Phe Ala Asp Pro Asp
        275                 280                 285
Arg Leu Asp Pro Asp Arg Ala Asp Ala Asp Arg Ala Leu Ser Ala Gln
    290                 295                 300
Arg Gly His Pro Gly Arg Leu Glu Glu Leu Val Val Val Leu Thr Thr
305                 310                 315                 320
Ala Ala Leu Arg Ser Val Ala Lys Ala Leu Pro Gly Leu Thr Ala Gly
                325                 330                 335
Gly Pro Val Val Arg Arg Arg Arg Ser Pro Val Leu Arg Ala Thr Ala
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340 345 350
His Cys Pro Val Glu Leu
355

<210> 17
<211> 422
<212> PRT
<213> Micromonospora megalomicea

<400> 17
Met Arg Val Val Phe Ser Ser Met Ala Ser Lys Ser His Leu Phe Gly
1 5 10 15
Leu Val Pro Leu Ala Trp Ala Phe Arg Ala Ala Gly His Glu Val Arg
20 25 30
Val Val Ala Ser Pro Ala Leu Thr Asp Asp Ile Thr Ala Ala Gly Leu
35 40 45
Thr Ala Val Pro Val Gly Thr Asp Val Asp Leu Val Asp Phe Met Thr
50 55 60
His Ala Gly Tyr Asp Ile Ile Asp Tyr Val Arg Ser Leu Asp Phe Ser
65 70 75 80
Glu Arg Asp Pro Ala Thr Ser Thr Trp Asp His Leu Leu Gly Met Gln
85 90 95
Thr Val Leu Thr Pro Thr Phe Tyr Ala Leu Met Ser Pro Asp Ser Leu
100 105 110
Val Glu Gly Met Ile Ser Phe Cys Arg Ser Trp Arg Pro Asp Trp Ser
115 120 125
Ser Gly Pro Gln Thr Phe Ala Ala Ser Ile Ala Ala Thr Val Thr Gly
130 135 140
Val Ala His Ala Arg Leu Leu Trp Gly Pro Asp Ile Thr Val Arg Ala
145 150 155 160
Arg Gln Lys Phe Leu Gly Leu Leu Pro Gly Gln Pro Ala Ala His Arg
165 170 175
Glu Asp Pro Leu Ala Glu Trp Leu Thr Trp Ser Val Glu Arg Phe Gly
180 185 190
Gly Arg Val Pro Gln Asp Val Glu Glu Leu Val Val Gly Gln Trp Thr
195 200 205
Ile Asp Pro Ala Pro Val Gly Met Arg Leu Asp Thr Gly Leu Arg Thr
210 215 220
Val Gly Met Arg Tyr Val Asp Tyr Asn Gly Pro Ser Val Val Pro Asp
225 230 235 240
Trp Leu His Asp Glu Pro Thr Arg Arg Arg Val Cys Leu Thr Leu Gly
245 250 255
Ile Ser Ser Arg Glu Asn Ser Ile Gly Gln Val Ser Val Asp Asp Leu
260 265 270
Leu Gly Ala Leu Gly Asp Val Asp Ala Glu Ile Ile Ala Thr Val Asp
275 280 285
Glu Gln Gln Leu Glu Gly Val Ala His Val Pro Ala Asn Ile Arg Thr
290 295 300
Val Gly Phe Val Pro Met His Ala Leu Leu Pro Thr Cys Ala Ala Thr
305 310 315 320
Val His His Gly Gly Pro Gly Ser Trp His Thr Ala Ala Ile His Gly
325 330 335
Val Pro Gln Val Ile Leu Pro Asp Gly Trp Asp Thr Gly Val Arg Ala
340 345 350
Gln Arg Thr Glu Asp Gln Gly Ala Gly Ile Ala Leu Pro Val Pro Glu
355 360 365
Leu Thr Ser Asp Gln Leu Arg Glu Ala Val Arg Arg Val Leu Asp Asp
370 375 380
Pro Ala Phe Thr Ala Gly Ala Ala Arg Met Arg Ala Asp Met Leu Ala
385 390 395 400
Glu Pro Ser Pro Ala Glu Val Val Asp Val Cys Ala Gly Leu Val Gly

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                405                     410                     415
Glu Arg Thr Ala Val Gly
            420

<210> 18
<211> 323
<212> PRT
<213> Micromonospora megalomicea

<400> 18
Met Ser Thr Asp Ala Thr His Val Arg Leu Gly Arg Cys Ala Leu Leu
 1               5                  10                  15
Thr Ser Arg Leu Trp Leu Gly Thr Ala Ala Leu Ala Gly Gln Asp Asp
            20                  25                  30
Ala Asp Ala Val Arg Leu Leu Asp His Ala Arg Ser Arg Gly Val Asn
        35                  40                  45
Cys Leu Asp Thr Ala Asp Asp Asp Ser Ala Ser Thr Ser Ala Gln Val
    50                  55                  60
Ala Glu Glu Ser Val Gly Arg Trp Leu Ala Gly Asp Thr Gly Arg Arg
65                  70                  75                  80
Glu Glu Thr Val Leu Ser Val Thr Val Gly Val Pro Pro Gly Gly Gln
                85                  90                  95
Val Gly Gly Gly Gly Leu Ser Ala Arg Gln Ile Ile Ala Ser Cys Glu
            100                 105                 110
Gly Ser Leu Arg Arg Leu Gly Val Asp His Val Asp Val Leu His Leu
        115                 120                 125
Pro Arg Val Asp Arg Val Glu Pro Trp Asp Glu Val Trp Gln Ala Val
    130                 135                 140
Asp Ala Leu Val Ala Ala Gly Lys Val Cys Tyr Val Gly Ser Ser Gly
145                 150                 155                 160
Phe Pro Gly Trp His Ile Val Ala Ala Gln Glu His Ala Val Arg Arg
                165                 170                 175
His Arg Leu Gly Leu Val Ser His Gln Cys Arg Tyr Asp Leu Thr Ser
            180                 185                 190
Arg His Pro Glu Leu Glu Val Leu Pro Ala Ala Gln Ala Tyr Gly Leu
        195                 200                 205
Gly Val Phe Ala Arg Pro Thr Arg Leu Gly Gly Leu Leu Gly Gly Asp
    210                 215                 220
Gly Pro Gly Ala Ala Ala Ala Arg Ala Ser Gly Gln Pro Thr Ala Leu
225                 230                 235                 240
Arg Ser Ala Val Glu Ala Tyr Glu Val Phe Cys Arg Asp Leu Gly Glu
                245                 250                 255
His Pro Ala Glu Val Ala Leu Ala Trp Val Leu Ser Arg Pro Gly Val
            260                 265                 270
Ala Gly Ala Val Val Gly Ala Arg Thr Pro Gly Arg Leu Asp Ser Ala
        275                 280                 285
Leu Arg Ala Cys Gly Val Ala Leu Gly Ala Thr Glu Leu Thr Ala Leu
    290                 295                 300
Asp Gly Ile Phe Pro Gly Val Ala Ala Ala Gly Ala Ala Pro Glu Ala
305                 310                 315                 320
Trp Leu Arg


<210> 19
<211> 247
<212> PRT
<213> Micromonospora megalomicea

<400> 19
Met Asn Thr Trp Leu Arg Arg Phe Gly Ser Ala Asp Gly His Arg Ala
 1               5                  10                  15
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Arg Leu Tyr Cys Phe Pro His Ala Gly Ala Ala Ala Asp Ser Tyr Leu
            20                  25                  30
Asp Leu Ala Arg Ala Leu Ala Pro Glu Val Asp Val Trp Ala Val Gln
        35                  40                  45
Tyr Pro Gly Arg Gln Asp Arg Arg Asp Glu Arg Ala Leu Gly Thr Ala
    50                  55                  60
Gly Glu Ile Ala Asp Glu Val Ala Ala Val Leu Arg Asp Leu Val Gly
65                  70                  75                  80
Glu Val Pro Phe Ala Leu Phe Gly His Ser Met Gly Ala Leu Val Ala
                85                  90                  95
Tyr Glu Thr Ala Arg Arg Leu Glu Ala Arg Pro Gly Val Arg Pro Leu
            100                 105                 110
Arg Leu Phe Val Ser Gly Gln Thr Ala Pro Arg Val His Glu Arg Arg
        115                 120                 125
Thr Asp Leu Pro Asp Glu Asp Gly Leu Val Glu Gln Met Arg Arg Leu
    130                 135                 140
Gly Val Ser Glu Ala Ala Leu Ala Asp Gln Gly Leu Leu Asp Met Ser
145                 150                 155                 160
Leu Pro Val Leu Arg Ala Asp His Arg Val Leu Arg Ser Tyr Ala Trp
                165                 170                 175
Gln Ala Gly Pro Pro Leu Arg Ala Gly Ile Thr Thr Leu Cys Gly Asp
            180                 185                 190
Thr Asp Pro Leu Thr Thr Val Glu Asp Ala Gln Arg Trp Leu Pro Tyr
        195                 200                 205
Ser Val Val Pro Gly Arg Thr Arg Thr Phe Pro Gly Gly His Phe Tyr
    210                 215                 220
Leu Ala Asp His Val Gly Glu Val Ala Glu Ser Val Ala Pro Asp Leu
225                 230                 235                 240
Leu Arg Leu Thr Pro Thr Gly
                245

<210> 20
<211> 189
<212> PRT
<213> Micromonospora megalomicea

<400> 20
Ile Arg Val Gln Asp Asp Asp Ala Asp Arg Leu Ser Arg Asp Glu Leu
1               5                   10                  15
Thr Ser Ile Ala Leu Val Leu Leu Leu Ala Gly Phe Glu Ala Ser Val
            20                  25                  30
Ser Leu Ile Gly Ile Gly Thr Tyr Leu Leu Leu Thr His Pro Asp Gln
        35                  40                  45
Leu Ala Leu Val Arg Lys Asp Pro Ala Leu Leu Pro Gly Ala Val Glu
    50                  55                  60
Glu Ile Leu Arg Tyr Gln Ala Pro Pro Glu Thr Thr Thr Arg Phe Ala
65                  70                  75                  80
Thr Ala Glu Val Glu Ile Gly Gly Val Thr Ile Pro Ala Tyr Ser Thr
                85                  90                  95
Val Leu Ile Ala Asn Gly Ala Ala Asn Arg Asp Pro Gly Gln Phe Pro
            100                 105                 110
Asp Pro Asp Arg Phe Asp Val Thr Arg Asp Ser Arg Gly His Leu Thr
        115                 120                 125
Phe Gly His Gly Ile His Tyr Cys Met Gly Arg Pro Leu Ala Lys Leu
    130                 135                 140
Glu Gly Glu Val Ala Leu Gly Ala Leu Phe Asp Arg Phe Pro Lys Leu
145                 150                 155                 160
Ser Leu Gly Phe Pro Ser Asp Glu Val Val Trp Arg Arg Ser Leu Leu
                165                 170                 175
Leu Arg Gly Ile Asp His Leu Pro Val Arg Pro Asn Gly
            180                 185
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<210> 21
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic nucleotide DNA duplex

<400> 21
taagaattcg gagatctggc ctcagctcta gac 33

<210> 22
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
<223> Complementary oligo

<400> 22
aattgtctag agctgaggcc agatctccga attcttaat 39

<210> 23
<211> 528
<212> DNA
<213> Micromonospora megalomicea

<400> 23
ttgcagcggt tgtcggtggc ggtgcgggag gggcgtcggg tgttgggtgt ggtggtgggt 60
tcggcggtga atcaggatgg ggcgagtaat gggttggcgg cgccgtcggg ggtggcgcag 120
cagcgggtga ttcggcgggc gtggggtcgt gcgggtgtgt cgggtgggga tgtgggtgtg 180
gtggaggcgc atgggacggg gacgcggttg ggggatccgg tggagttggg ggcgttgttg 240
gggacgtatg gggtgggtcg gggtggggtg ggtccggtgg tggtgggttc ggtgaaggcg 300
aatgtgggtc atgtgcaggc ggcggcgggt gtggtgggtg tgatcaaggt ggtgttgggg 360
ttgggtcggg ggttggtggg tccgatggtg tgtcggggtg ggttgtcggg gttggtggat 420
tggtcgtcgg gtgggttggt ggtggcggat ggggtgcggg ggtggccggt gggtgtggat 480
ggggtgcgtc ggggtggggt gtcggcgttt ggggtgtcgg ggacgaat 528

<210> 24
<211> 528
<212> DNA
<213> Micromonospora megalomicea

<400> 24
ctgcagcggt tgtcggtggc ggtgcgggag gggcgtcggg tgttgggtgt ggtggtgggt 60
tcggcggtga atcaggatgg ggcgagtaat gggttggcgg cgccgtcggg ggtggcgcag 120
cagcgggtga ttcggcgggc gtggggtcgt gcgggtgtgt cgggtgggga tgtgggtgtg 180
gtggaggcgc atgggacggg gacgcggttg ggggatccgg tggagttggg ggcgttgttg 240
gggacgtatg gggtgggtcg gggtggggtg ggtccggtgg tggtgggttc ggtgaaggcg 300
aatgtgggtc atgtgcaggc ggcggcgggt gtggtgggtg tgatcaaggt ggtgttgggg 360
ttgggtcggg ggttggtggg tccgatggtg tgtcggggtg ggttgtcggg gttggtggat 420
tggtcgtcgg gtgggttggt ggtggcggat ggggtgcggg ggtggccggt gggtgtggat 480
ggggtgcgtc ggggtggggt gtcggcgttt ggggtgtcgg ggacgaat 528

<210> 25
<211> 528
<212> DNA
<213> Micromonospora megalomicea

<220>

<221> misc_feature
<222> (1)...(528)
<223> Sequence with codon changes as described in the
specification at page 99, line 22 thru 101, line 23

<400> 25
ctgcagcgcc tctccgtcgc cgtccgcgag ggccgccgag tcctcggcgt cgtcgtcggc 60
tcggccgtca accaagacgg cgcgtcaaac ggcctcgccg cgccctccgg cgtcgcccag 120
cagcgcgtca tacgccgcgc gtggggacgc gccggagtat cgggcggcga cgtcggagtc 180
gtcgaggccc acggcaccgg cacccgcctc ggggatcccg tcgagctggg cgccctcctg 240
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aacgtcggcc acgtccaggc cgcggccggc gtcgtcgggg tcatcaaggt cgtcctcggc 360
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tggtcgtccg gcggcctggt cgtcgcggac ggggtccgcg gctggccggt cggcgtcgac 480
ggcgtccgcc ggggcggcgt ctcggcgttc ggcgtcagcg ggacgaat 528

<210> 26
<211> 291
<212> DNA
<213> Micromonospora megalomicea

<400> 26
ggtggagtgt gatgcggtgg tgtcgtcggt ggtggggttt tcggtgttgg gggtgttgga 60
gggtcggtcg ggtgcgccgt cgttggatcg ggtggatgtg gtgcagccgg tgttgttcgt 120
ggtgatggtg tcgttggcgc ggttgtggcg gtggtgtggg gttgtgcctg cggcggtggt 180
gggtcattcg caggggggaga tcgcggcggc ggtggtggcg gggtgttgt cggtgggtga 240
tggtgcgcgg gtggtggcgt tgcgggcgcg ggcgttgcgg gcgttggccg g 291

<210> 27
<211> 291
<212> DNA
<213> Micromonospora megalomicea

<400> 27
ggtggagtgt gatgcggtgg tgtcgtcggt ggtggggttt tcggtgttgg gggtgttgga 60
gggtcggtcg ggtgcgccgt cgttggatcg ggtggatgtg gtgcagccgg tgttgttcgt 120
ggtgatggtg tcgttggcgc ggttgtggcg gtggtgtggg gttgtgcctg cggcggtggt 180
gggtcattcg caggggggaga tcgcggcggc ggtggtggcg gggtgttgt cggtgggtga 240
tggtgcgcgg gtggtggcgt tgcgggcgcg ggcgttgcgg gcgttggccg g 291

<210> 28
<211> 291
<212> DNA
<213> Micromonospora megalomicea

<220>
<221> misc_feature
<222> (1)...(291)
<223> Sequence with codon changes as described in the
specification at page 99, line 22 thru page 101, line 23

<400> 28
cgtggagtgc gatgcggtcg tgtcgagcgt cgtcggcttc agcgtgctgg gcgtcctgga 60
gggccgcagc ggcgccccga gcctggaccg cgtcgacgtg gtccagccgg tcctgttcgt 120
ggtcatggtc agcctggccc gcctgtggcg ctggtgcggc gtggtcccgg ccgccgtggt 180
cggccacagc cagggcgaga tcgccgccgc ggtcgtggcc ggcgtcctga gcgtcggcga 240
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<210> 29
<211> 24
<212> DNA

<213> Artificial Sequence

<220>
<223> PCR primer

<400> 29
gaacaactcc tgtctgcggc cgcg 24

<210> 30
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR primer

<400> 30
cggaattctc tagagtcacg tctccaaccg cttgtcgagg 40

<210> 31
<211> 51
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR primer

<400> 31
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<210> 32
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR primer

<400> 32
aacgcctccc aggagatctc cagca 25

<210> 33
<211> 16
<212> DNA
<213> Artificial Sequence

<220>
<223> Oligo

<400> 33
aattcatagc ctaggt 16

<210> 34
<211> 16
<212> DNA
<213> Artificial Sequence

<220>
<223> Oligo

<400> 34

INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/US 00/27433

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/52 C12N15/53 C12N15/54 C12N15/61 C12N15/62
C12N9/04 C12N9/10 C12N9/90 C12P19/62

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EMBL, EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 23630 A (ABBOTT LAB) 3 July 1997 (1997-07-03) the whole document claims 1-22 figures 1-3 ---	1-12,14, 18,19
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 June 2001	09/07/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	van de Kamp, M

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	NAKAGAWA A ET AL.: "Structure and stereochemistry of macrolides" MACROLIDE ANTIBIOTICS. OMURA S (ED.). PUBLISHER: ACADEMIC, ORLANDO, FLORIDA, 1984, pages 37-84, XP001006199 page 46, line 25 -page 48, line 4	

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A	LIU H -W ET AL: "Pathways and mechanisms in the biogenesis of novel deoxysugars by bacteria" ANNUAL REVIEW OF MICROBIOLOGY, vol. 48, 1994, pages 223-256, XP002061259 page 234, line 24 -page 237, line 9; figures 8,9	1,5-12, 19
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P,X	WO 00 00500 A (LEADLAY PETER FRANCIS ;CORTES JESUS (GB); STAUNTON JAMES (GB); BIO) 6 January 2000 (2000-01-06) claim 24	14

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/27433

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO 00 63361 A (KOSAN BIOSCIENCES INC) 26 October 2000 (2000-10-26) page 9, line 3-9 page 14, line 26 -page 16, line 2 claim 3 -----	1-13, 18-20

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Information on patent family members

International Application No
PCT/US 00/27433

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